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STUDIES ON COMPLEMENT FIXATION.

I. The Rate of Fixation of Complement at Different Temperatures.

By R. L. KAHN, Sc.D.

(From the Bureau of Laboratories of Michigan Department of Health, Lansing.)

(Received for publication, April 1, 1921.)

INTRODUCTION.

A more complete understanding of the complement fixation phenomenon will perhaps ultimately be achieved by the employment of purified proteins as antigens. The mass of accumulated studies of this phenomenon with organ extracts as antigens may be said to apply largely to the Wassermann test. For, it is questionable whether a principle derived with a non-specific organ extract antigen can apply in all cases to specific antigen-antibody complexes. These Wassermann studies might indeed explain the limited application of the complement fixation test to the diagnosis of diseases other than syphilis and to the identification of unknown organisms.

The antigen, however, is not the only element in which the Wassermann test varies from specific complement fixation tests. Recent studies reported from this laboratory (1) indicate that the behavior of the so called syphilitic complement-fixing antibody towards heat is markedly different from the behavior towards this agent of the complement-fixing antibody found in rabbits after immunization with purified proteins. The former is completely destroyed at temperatures ranging between 62° and 65°C., while the thermal destructive point of the latter is in the neighborhood of 80°C.

The complement fixation reaction, furthermore, is so complex, that the reduction of even one of its ingredients to the simplest elements ought to help in its interpretation. And although chemically the protein molecule is a huge structure indeed, biologically it stands out

as the simplest possible element capable of producing complement-fixing antibodies (2).

These considerations led us to undertake a series of investigations of the complement fixation phenomenon with purified proteins as antigens. These studies are still being continued and are being extended also to organ extracts with syphilitic sera as well as bacterial antigens with their specific antisera with a view of finding whether the principles developed by means of protein antigens are applicable also to practical tests.

The factors governing the fixation of complement were chosen as the subject of the first paper of this series, because of their fundamental importance in all complement fixation studies. Investigations on the effects of temperature and time on the fixation of complement have heretofore been restricted largely to the Wassermann test. The literature covering this field will be presented in a forthcoming paper in which the rate of fixation of complement in the Wassermann test will be considered. In this connection, the work of Dean (3) needs quoting inasmuch as in his studies on the effect of temperature on the fixation of complement, he employed a specific antigen—horse serum. This investigator employed a 1 hour fixation period at temperatures of 0°, 17°, and 37° C., and found uniformly more complement fixed at 0° C. than at 17° C. and more at 17° C. than at 37° C. This work is of especial interest in view of the fact that in accordance with the prevailing opinion (4, 5) the phenomenon of fixation of complement takes place far more readily at water bath than at colder temperatures.

EXPERIMENTAL.

The plan of these studies was, first to establish the presence of specific complement-fixing antibodies in the sera of rabbits previously immunized with purified proteins; second, to determine by means of complement fixation tests with the immune sera and specific protein antigens, the rate of fixation of complement, and to what extent this rate is affected by different temperatures.

The antigens employed were edestin, obtained from hemp-seed, and phaseolin, obtained from the kidney bean.[1] Three fixation tem-

[1] These were kindly furnished by Dr. Thomas B. Osborne of the Connecticut Agricultural Experiment Station.

peratures were adhered to: ice box (8–12°C.), room (18–23°C.), and water bath (37.5°C.). The degree of fixation was determined every 15 minutes during the 1st hour, at half hour periods during the 2nd hour, followed by determinations every hour until 5 or 6 hours had passed.

Methods of Immunization.

Three different methods of immunization were resorted to in order to vary the antibody content in the rabbit sera. Rabbit A received five intravenous injections of edestin at 48 hour intervals. The quantities of protein injected were 50, 75, 100, 125, and 150 mg. respectively. Rabbit B received five intraperitoneal injections of phaseolin at 48 hour intervals, the quantities being 100, 150, 200, 250, and 300 mg. respectively. Rabbit C received three intraperitoneal injections of phaseolin at 24 hour intervals, the quantities being 100, 150, and 200 mg. respectively.

Both edestin and phaseolin are insoluble in water but soluble in weak alkaline solutions. This necessitated the addition of a few drops of 0.1N sodium hydroxide to the proteins in order to get them in solution. This was done in each case just before the injection.

Procedure of the Complement Fixation Test.

The sheep cell system was employed. All ingredients entering into the test were used in 0.1 cc. quantities except the immune rabbit serum which was graded from 0.01 to 0.0001 cc. Two units of amboceptor, two units of complement, and from two to ten units of antigen were employed.

Sheep Cells.—Sheep were bled from the jugular vein into sterile bottles containing glass beads. After defribrination by shaking, the blood was washed four times with saline solution. The final centrifugation was carried out for 14 minutes at 1,500 revolutions per minute. 5 per cent was the strength of the standard suspension.

Hemolysin.—This was prepared in the usual manner by injecting rabbits with washed sheep cells. The unit of hemolysin was determined by titrating a series of dilutions of hemolysin serum with 0.1 cc. quantities of 1:10 pooled guinea pig complements and 0.1 cc. quantities of the standard sheep cell suspension and reading the unit

after 15 minutes incubation in the water bath (6). The serum dilution aimed at was one which contained two units of hemolysin in 0.1 cc.

Complement.—Large sized guinea pigs were bled directly from the heart. The serum was drawn after permitting the blood to remain for about 20 hours in the ice box. Pooled serum from four to five guinea pigs was used with each experiment. The unit of complement was obtained by titrating gradations of 0.1 to 0.01 cc. of both single and pooled complements with two units (0.1 cc.) of amboceptor and 0.1 cc. quantities of sheep cells. The unit in this case also was read after 15 minutes incubation in the water bath.

Protein antigens.—These were prepared by weighing 10 mg. of the protein and dissolving it in 10 cc. of 0.001N sodium hydroxide solution to which was added 0.05 cc. of 0.1N sodium hydroxide. The alkali used, represents approximately the smallest quantity necessary to get the proteins into solution. 1 cc. of this antigen solution was mixed with 9 cc. of salt solution and 0.1 cc. of this final solution, corresponding to 0.01 mg. of antigen, was used in the tests. Antigenic titrations of this solution with 0.01 cc. of specific immune serum and two units of complement showed, after primary incubation for 4 hours in the ice box, complete fixation of complement with 0.02 cc. (0.002 mg. of protein) and partial fixation with 0.01 cc. (0.001 mg. of protein). Tests for anticomplementary and hemolytic properties of these protein antigens showed that neither possessed these properties with five times the quantity (0.5 cc.) of antigen used in the tests.

Immune Serum.—The immune serum was obtained in each case by bleeding the rabbits from the marginal ear vein and separating the serum after permitting the blood to remain for several hours in the ice box. Both the edestin and phaseolin rabbits showed the presence of specific complement-fixing antibodies about 10 days after the last injection of protein. The sera were inactivated in each case for 30 minutes at 56° C. before using, and were employed in the following dilutions in the tests: 0.01, 0.007, 0.004, 0.003, 0.002, 0.001, 0.0005, 0.0003, 0.0001 cc.

Complement Fixation Tests.—The tests were carried out in the usual manner, regular Wassermann tubes being employed with the various gradations of serum, 0.1 cc. (0.01 mg.) of the protein antigen, 0.1 cc. (two units) of complement, and 0.1 cc. of saline solution. After

a given fixation period 0.1 cc. of the standard sheep cell suspension and 0.1 cc. of hemolysin (two units) were added and incubated in the water bath at 37.5° C. for about 15 minutes when the serum and antigen controls would be completely hemolyzed. All readings were made after keeping the tubes in the ice box over night. The temperatures and periods of fixation were the only variables in each test.

Effect of Temperature on the Rate of Fixation of Complement.

Contrary to the accepted views that the fixation of complement takes place far more rapidly at water bath than at a lower temperature, preliminary experiments with the rabbit sera and specific protein antigens indicated no marked difference in the degree of fixation between water bath (37.5° C.) and ice box (8–12°C.) fixation temperatures. This indeed is the reason why no attempt was made in these studies to use precise temperatures in the case of ice box and room temperature fixation. Table I gives an outline of one of these experiments with edestin immune serum. 15 minutes was the time interval chosen for fixation during the 1st hour, and multiples of 15 minutes for the succeeding hours—up to 5 or 6. It is evident from this table that the degree of fixation as measured by the increase in number of positive signs is approximately the same whether ice-box or water bath fixation is employed. There is indeed a tendency for stronger fixation in the ice box than in the water bath.

The fixation experiments at water bath and room temperatures were not extended beyond 2 hours in view of the marked deterioration of complement which takes place after prolonged exposure at these temperatures.

Text-fig. 1 shows the nature of the curve with the degree of fixation as determined by the increase in number of positive signs as the ordinate and time of fixation as the abscissa.

Table II and Text-fig. 2 represent a similar experiment with phaseolin immune serum.

Tables III and IV and Text-figs. 3 and 4 represent fixation experiments carried out at water bath, room, and ice box temperatures. The tendency for somewhat stronger fixation in the ice box is shown by

these experiments; also the fixation at room temperature approximates more closely fixation in the ice box than that in the water bath.

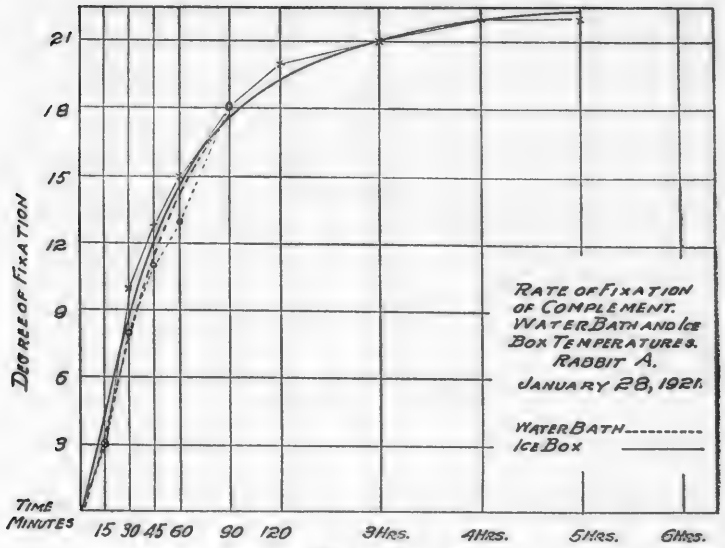
Text-figs. 5 and 6 show a marked similarity with the previous experiments and indicate to what extent the curves of fixation approximate

TABLE I.

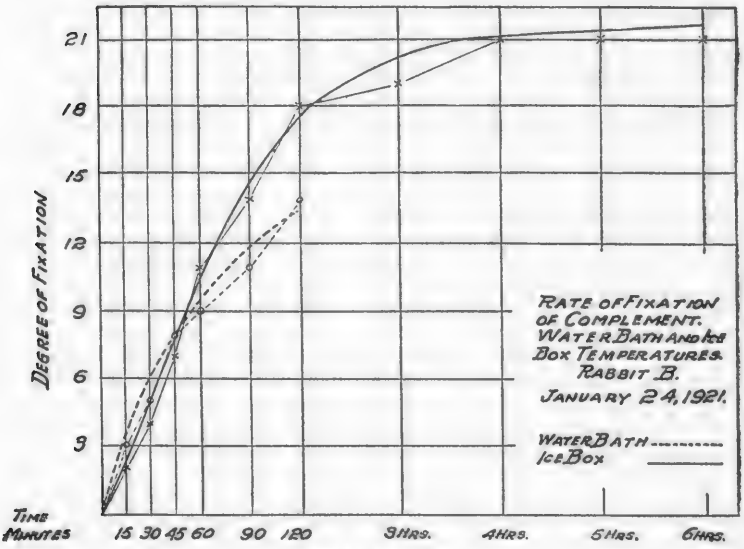
Rate of Increase of Fixation of Complement at Water Bath and Ice Box Temperatures.

Fixation.		Edestin immune serum, Rabbit A.									No. of positive signs, denoting degree of fixation.
Period.	Temperature.	0.01 cc.	0.007 cc.	0.004 cc.	0.003 cc.	0.002 cc.	0.001 cc.	0.0005 cc.	0.0003 cc.	0.0001 cc.	
min.											
15	Water bath.	+	+	+	−	−	−	−	−	−	3
	Ice box.	+	+	+	−	−	−	−	−	−	3
30	Water bath.	+++	++	+	+	+	−	−	−	−	8
	Ice box.	+++	+++	++	+	+	−	−	−	−	10
45	Water bath.	++++	+++	++	+	+	−	−	−	−	11
	Ice box.	++++	++++	++	+	+	+	−	−	−	13
60	Water bath.	++++	+++	++	++	+	+	−	−	−	13
	Ice box.	++++	++++	+++	++	+	+	−	−	−	15
90	Water bath.	++++	++++	++++	+++	++	+	−	−	−	18
	Ice box.	++++	++++	++++	+++	++	+	−	−	−	18
120	Water bath.	++++	++++	++++	++++	+++	+	−	−	−	20
	Ice box.	++++	++++	++++	++++	+++	+	−	−	−	20
180	" "	++++	++++	++++	++++	+++	++	−	−	−	21
240	" "	++++	++++	++++	++++	++++	+	+	−	−	22
300	" "	++++	++++	++++	++++	++++	+	+	−	−	22

one another when the concentration of antibodies is about the same. The lesser degree of fixation at water bath temperature shown in Text-figs. 3 and 5 is difficult to explain in view of the fact that the sera of the same rabbit did not show this tendency to the same degree in Text-fig. 1.



TEXT-FIG. 1.



TEXT-FIG. 2.

Text figure 7 is given as an illustration of the nature of the curve with a weakly positive serum.

TABLE II.

Rate of Increase of Fixation of Complement at Water Bath and Ice Box Temperatures.

Fixation.		Phaseolin immune serum, Rabbit B.									No. of positive signs, denoting degree of fixation.
Period.	Temperature.	0.01 cc.	0.007 cc.	0.004 cc.	0.003 cc.	0.002 cc.	0.001 cc.	0.0005 cc.	0.0003 cc.	0.0001 cc.	
min.											
15	Water bath.	++	+	−	−	−	−	−	−	−	3
	Ice box.	+	+	−	−	−	−	−	−	−	2
30	Water bath.	+++	+	+	−	−	−	−	−	−	5
	Ice box.	++	+	+	−	−	−	−	−	−	4
45	Water bath.	++++	++	+	+	−	−	−	−	−	8
	Ice box.	+++	++	+	+	−	−	−	−	−	7
60	Water bath.	++++	+++	+	+	−	−	−	−	−	9
	Ice box.	++++	+++	++	+	+	−	−	−	−	11
90	Water bath.	++++	++++	++	+	−	−	−	−	−	11
	Ice box.	++++	++++	+++	++	+	−	−	−	−	14
120	Water bath.	++++	++++	+++	++	+	−	−	−	−	14
	Ice box.	++++	++++	++++	+++	++	+	−	−	−	18
180	" "	++++	++++	++++	++++	++	+	−	−	−	19
240	" "	++++	++++	++++	++++	++++	+	−	−	−	21
300	" "	++++	++++	++++	++++	++++	+	−	−	−	21
360	" "	++++	++++	++++	++++	++++	+	−	−	−	21

A consideration of the experimental date presented in this paper indicates that the affinity for complement of specific protein antigen-antibody complexes is extremely marked, fixation of complement taking place equally as well at 8–12°C. as at 37.5°C. Theoretically this is suggestive of the close relation between the phenomenon of

precipitation and that of complement fixation, the former also taking place at cold temperatures.

TABLE III.

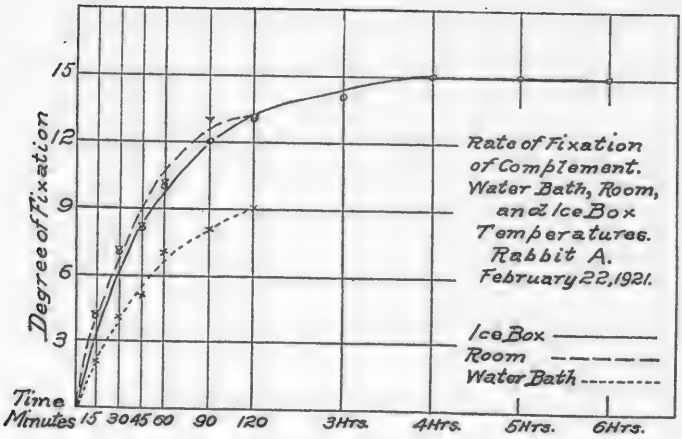
Rate of Increase of Fixation of Complement at Water Bath, Room, and Ice Box Temperatures.

Fixation.		Edestin immune serum, Rabbit A								No. of positive signs, denoting degree of fixation.
Period.	Temperature.	0.01 cc.	0.007 cc.	0.004 cc.	0.003 cc.	0.002 cc.	0.001 cc.	0.0005 cc.	0.0003 cc.	0.0001 cc.	
min.											
15	Water bath.	+	+	−	−	−	−	−	−	−	2
	Room.	++	+	+	−	−	−	−	−	−	4
	Ice box.	++	+	+	−	−	−	−	−	−	4
30	Water bath.	++	+	+	−	−	−	−	−	−	4
	Room.	+++	++	+	+	−	−	−	−	−	7
	Ice box.	+++	++	+	+	−	−	−	−	−	7
45	Water bath.	++	++	+	−	−	−	−	−	−	5
	Room.	+++	+++	+	+	−	−	−	−	−	8
	Ice box.	+++	+++	+	+	−	−	−	−	−	8
60	Water bath.	+++	++	+	+	−	−	−	−	−	7
	Room.	++++	+++	++	+	−	−	−	−	−	10
	Ice box.	++++	+++	++	+	−	−	−	−	−	10
90	Water bath.	+++	+++	+	+	−	−	−	−	−	8
	Room.	++++	++++	+++	+	+	−	−	−	−	13
	Ice box.	++++	++++	+++	+	−	−	−	−	−	12
120	Water bath.	+++	+++	++	+	−	−	−	−	−	9
	Room.	++++	++++	+++	+	+	−	−	−	−	13
	Ice box.	++++	++++	+++	+	+	−	−	−	−	13
180	" "	++++	++++	+++	++	+	−	−	−	−	14
240	" "	++++	++++	+++	++	+	+	−	−	−	15
300	" "	++++	++++	+++	++	+	+	−	−	−	15
360	" "	++++	++++	+++	++	+	+	−	−	−	15

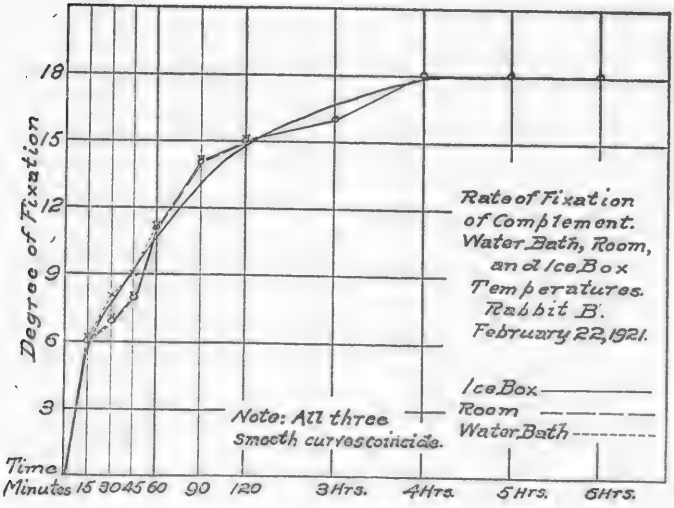
TABLE IV.

Rate of Increase of Fixation of Complement at Water Bath, Room, and Ice Box Temperatures.

Fixation.		Phaseolin immune serum, Rabbit B									No. of positive signs, denoting degree of fixation.
Period.	Temperature.	0.01 cc.	0.007 cc.	0.004 cc.	0.003 cc.	0.002 cc.	0.001 cc.	0.0005 cc.	0.0003 cc.	0.0001 cc.	
min.											
15	Water bath.	+++	++	+	−	−	−	−	−	−	6
	Room.	+++	++	+	−	−	−	−	−	−	6
	Ice box.	+++	++	+	−	−	−	−	−	−	6
30	Water bath.	+++	+++	+	+	−	−	−	−	−	8
	Room.	+++	++	+	+	−	−	−	−	−	7
	Ice box.	+++	++	+	+	−	−	−	−	−	7
45	Water bath.	++++	+++	+	+	−	−	−	−	−	9
	Room.	+++	+++	+	+	−	−	−	−	−	8
	Ice box.	+++	+++	+	+	−	−	−	−	−	8
60	Water bath.	++++	++++	++	+	−	−	−	−	−	11
	Room.	++++	++++	++	+	−	−	−	−	−	11
	Ice box.	++++	++++	++	+	−	−	−	−	−	11
90	Water bath.	++++	++++	+++	++	+	−	−	−	−	14
	Room.	++++	++++	+++	++	+	−	−	−	−	14
	Ice box.	++++	++++	+++	++	+	−	−	−	−	14
120	Water bath.	++++	++++	+++	++	+	+	−	−	−	15
	Room.	++++	++++	+++	++	+	+	−	−	−	15
	Ice box.	++++	++++	+++	++	+	+	−	−	−	15
180	" "	++++	++++	+++	+++	+	+	−	−	−	16
240	" "	++++	++++	++++	+++	++	+	−	−	−	18
300	" "	++++	++++	++++	+++	++	+	−	−	−	18
360	" "	++++	++++	++++	+++	++	+	−	−	−	18

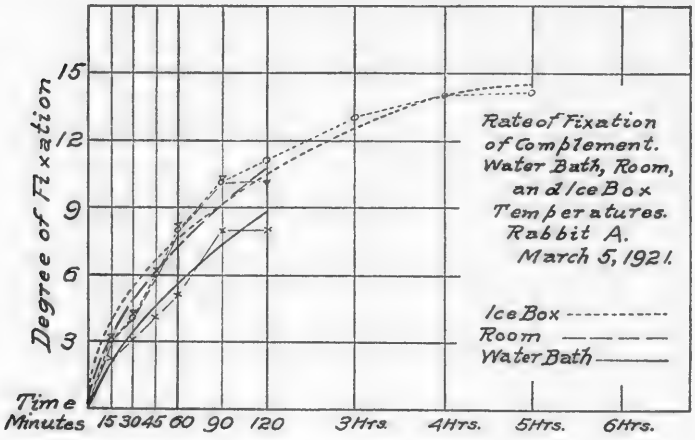


TEXT-FIG. 3.

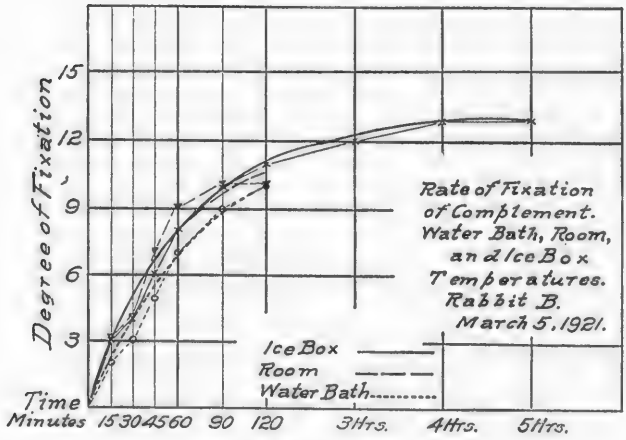


TEXT-FIG. 4.

These experiments also indicate that the rate of fixation of complement is directly proportional to the concentration of antibodies in



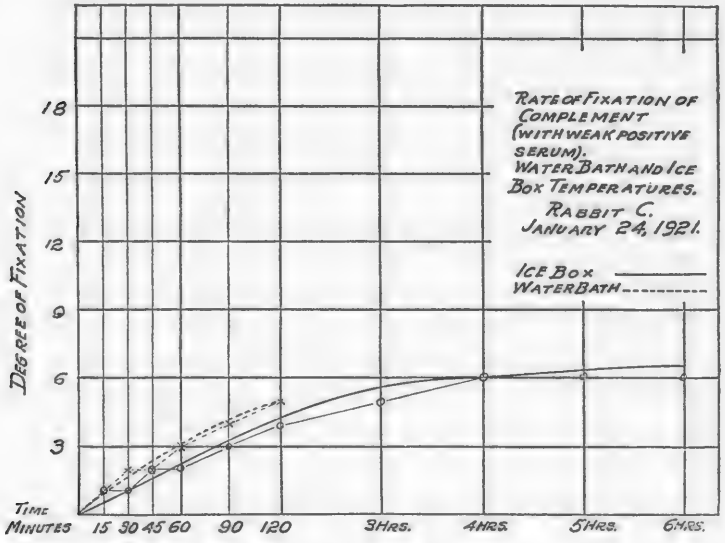
TEXT-FIG. 5.



TEXT-FIG. 6.

the immune serum. This is evident by observing the various curves; the greater the concentration of antibodies, the steeper the curve.

The rate of fixation of complement at different temperatures with various Wassermann antigens and syphilitic sera will be reported in forthcoming studies.



TEXT-FIG. 7.

CONCLUSIONS.

It is shown by complement fixation studies with protein antigens and specific immune rabbit sera that the rate of fixation of complement is determined by the concentration of antibodies in the immune sera, that the greater part of fixation of complement takes place during the 1st hour, and that fixation is practically completed at the end of 4 hours at ice box temperature.

It is further shown that the rate of fixation of complement is practically the same at ice box, room, and water bath temperatures, the tendency being for slightly stronger fixation at ice box temperature.

BIBLIOGRAPHY.

1. Kahn, R. L., On the thermostability of complement fixing antibodies resulting from protein immunization, *Proc. Soc. Exp. Biol. and Med.*, 1921, xviii, 171.
2. Kahn, R. L., and McNeil, A., Complement fixation with protein substances, *J. Immunol.*, 1918, iii, 277.
3. Dean, H. R., The influence of temperature on the fixation of complement, *J. Path. and Bact.*, 1917, xxi, 193.
4. Noguchi, H., Influence of temperature upon the velocity of the complement fixation reaction in syphilis, *J. Exp. Med.*, 1918, xxviii, 297.
5. Kolmer, J. A., Rule, A. M., and Yagle, E. M., Studies in the standardization of the Wassermann reaction. XVI. The influence of temperature and duration of primary incubation upon the velocity and amount of complement fixation in syphilis with different organ extracts (antigens), *J. Syph.*, 1921, V, 44.
6. Kahn, R. L., Complement *vs.* amboceptor titrations in the Wassermann test, *J. Lab. and Clin. Med.*, 1920–21, vi, 153.

MEASUREMENT OF THE GROWTH OF TISSUES IN VITRO.

By ALBERT H. EBELING, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, March 29, 1921.)

Two fragments of the same tissue placed in identical media do not always grow at the same rate. Therefore, an acceleration or a retardation of the rate of growth of a tissue placed in an experimental medium cannot be considered as due necessarily to the action of the medium. It may be a mere accident. In order that the differences in the rate of growth of tissues, placed in media of different composition, may be attributed to an action of the medium, it must be ascertained whether these tissues would grow at the same rate, if placed in identical media. In the course of many experiments, it was attempted to find the conditions under which two parts of the same fragment of tissue cultivated in identical media would increase with the same velocity, and to develop a technique by which the increment could be accurately measured.

I. Technique for Obtaining the Growth of Two Fragments of Tissue at the Same Rate.

It is already known that cultures of tissues, made under certain conditions, generally grow at about the same rate.[1] But in several instances, it was found that the differences in area observed after 48 hours reached 20 or even 50 per cent. The causes of these variations were carefully investigated and the reason for the inequality of the growth could always be discovered. Progressively, a number of minute details of the technique were modified and improved, and a method which renders the growth of two fragments of tissue more nearly equal was developed.

[1] Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 534–535.

1. Selection of the Tissue.—Connective tissue cells, the growth of which was the subject of the experiments, were obtained from embryonic tissues and from an old strain of fibroblasts. The growth of embryonic tissues is not regular. Moreover, it is almost impossible to cut such fragments in strictly equal parts. However, the heart of chick embryo was found to grow more regularly than other tissues. After 48 hours, a dense ring of fibroblasts has formed around the original fragment and beyond the ring are scattered fusiform or multipolar cells. Sometimes a more or less irregular layer of endothelial cells spreads into the plasma. If the presence of the peripheral cells is disregarded, and the dense ring of fibroblasts is alone taken into consideration, the area of new growth can be measured with some accuracy. The width of this ring of densely packed cells does not vary markedly, and the uniformity of its thickness can easily be ascertained. The growth consists not only of the migration and the multiplication of the cells in the medium, but also of the increase in mass of the original fragment. The increase in area can easily be measured. But, as the tissue is completely opaque, no appreciation of its thickness can be reached. However, if the fragments selected are identical, not only in surface but also in thickness, they grow at about the same rate. This condition may be obtained by the following technique: A heart taken from a chick embryo about 12 days old is opened and washed in Ringer solution. Afterwards, the walls of the ventricles are placed on a glass plate in a little Ringer solution, and square fragments are cut with a sharp cataract knife. When such a fragment is bisected, both parts are of the same thickness, and if their surface is about equal, they grow at the same rate. Often the limits of the new growth and of the primitive fragment become indistinct, and this prevents accurate appreciation of the width of the growth. Although the growth of embryonic heart is more regular than that of other organs, it is still unfitted for accurate measurement. Whenever possible, a strain of connective tissue adapted to life *in vitro* should be employed.

The tissues are taken from stock cultures of a strain of fibroblasts kept for 9 years in plasma and embryonic juices. The area of the fragment must be about 3 by 5 mm. As the tissue is not entirely opaque to light, it is easy to see whether or not its density is uniform.

Zones of greater density, where the cells are often necrotic, can be detected easily by their complete opacity to transmitted light, and they appear as white spots when they are seen by refracted light. The cultures showing these local thickenings are discarded, unless the area of greater density is symmetrical, and can be divided into two equal parts. It is very much easier to select tissues of uniform density when the old strain is used than when the cultures are made of embryonic heart. Another advantage of the old strain is that the edges of the original fragment generally remain visible for 48 hours, and that the inner and outer limits of the new tissue can be recognized. The area of the original fragment remains constant and the entire growth consists of the ring of new tissue in the medium. A third advantage of the old strain of connective tissue is that only fibroblasts grow from it. There are no ameboid cells or layers of endothelial cells, or scattered cells at the periphery of the thick ring of fibroblasts. The outline of the new growth is sharp and can be traced easily. While our experiments were made with a 9 year old strain of fibroblasts, a younger strain can be used with the same results. By placing a fragment of embryonic heart in plasma and embryonic juice, and by resecting the peripheral growth of fibroblasts when it has become very extensive, and cultivating it again, one can obtain an excellent strain of connective tissue. After ten or twelve passages, fibroblasts are practically in pure culture, grow at a regular rate, and can be used for a quantitative study of the growth of connective tissue.

2. *Preparation of the Tissue.*—The pieces of tissue used for an experiment always consisted of two or four parts of the same fragment. Fragments coming from different cultures may present differences in activity or in structual conditions which would render the rate of growth unequal. When a old strain of fibroblasts is used, the tissue is extirpated from the coagulum by four neat cuts of a sharp cataract knife, made through the peripheral growth. No part of the coagulum not yet invaded by the cells must be left around the tissue. If the incisions are not very sharp, the coagulated plasma may become folded over the cut edge, or a tractus of fibrin still adherent to the remaining coagulum may produce a dislocation of the fibrin network when the tissue is removed from the culture. When the coagulum

has been severed by the knife, the edge of the wound is slightly pushed aside with the blade, in order to make sure that no tractus of fibrin has been left adherent. After the fragment is completely detached from the surrounding coagulum, it is taken on the edge of the knife and placed in Ringer solution. It is examined again in order to make sure that the edges are not folded and that no fragment of mica is adherent to the tissue. With the cataract knife, it is divided into two parts, as equal as possible. It takes a great deal of practice to learn to divide the fragment in the proper way. If the two parts so obtained are not equal in area and in thickness, or if their edges are dilacerated, they must be discarded.

3. Preparation of the Medium.—The preparation of plasma, Ringer solution, and tissue juices has already been described.[2] It was found that still more care should be given to the possible changes of the hydrogen ion concentration of these different substances, and to the impurities which may come from the chemicals used in the salt solutions. Distilled water is redistilled in glass, and then its pH is determined. The purest chemicals are used for Ringer solution. The solutions are kept in Pyrex glassware, or in ordinary glass coated with paraffin. The pipettes are also tested in order to be sure that the alakalinity of the fluid is not increased by their contact. The H ion concentration of the serum and tissue extract may undergo progressive changes. Therefore, they should be carefully tested. Generally, while the pH of embryonic tissue juice is about 7.2 at the time of the preparation, it drops after a few days to 6.5, and if in contact with the pulp remains at that figure for a long time. The pH of plasma remains constant for several weeks. There are other changes which occur in the course of time in serum, and especially in tissue juices and extract, which make them less efficient in promoting growth and which are independent of the changes in the hydrogen ion concentration. It is, therefore, safer not to keep these substances for more than a few days in cold storage.

4. Preparation of the Cultures.—The fluids composing the medium are placed on the cover-glasses at the same time and thoroughly mixed. Plasma should be placed first on the surface of the glass and spread over it, and then the other substancess should be added. This detail

[2] Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 532–533.

insures against subsequent partial or total detachment of the clot. Then the tissue fragments are taken from the Ringer solution and placed in the medium. It is important that both media should be not only of the same composition, but also of the same volume and area. The growth of a fragment of tissue in identical volumes of fluid differs when the fluid is spread at the surface of the cover-glass and when it is left in a thick drop. In order that both media may be spread equally, the cover-glass is put on a piece of paper on which a circle has been traced. After the plasma is dropped on the glass, it is spread with the point of a knife in such a way that it covers the subjacent circle. This technique allows the surface of the medium to be identical in both cultures. As cover-glasses are often of varied alkalinity, mica is substituted for glass, or both cultures are made on the same long cover-glass, in order that the conditions may be identical. Immediately after the fragments of tissue are imbedded in the medium, a hollow slide covered with a little vaseline is inverted over the cover-glasses. Care is taken that the capacity of the hollow slides is identical in both cultures. If one is larger, the evaporation of the medium is greater and its concentration increases. It is well known that a change in the concentration of the medium will modify the extent of the growth. In order to eliminate completely this cause of error, both cultures may be made on the same cover-glass and inverted over an oblong hollow slide. The cultures are then sealed at the same time, and placed in the incubator simultaneously and close together, in order that they may not be subjected to different temperatures. They are allowed to grow for 48 hours.

II. Measurement.

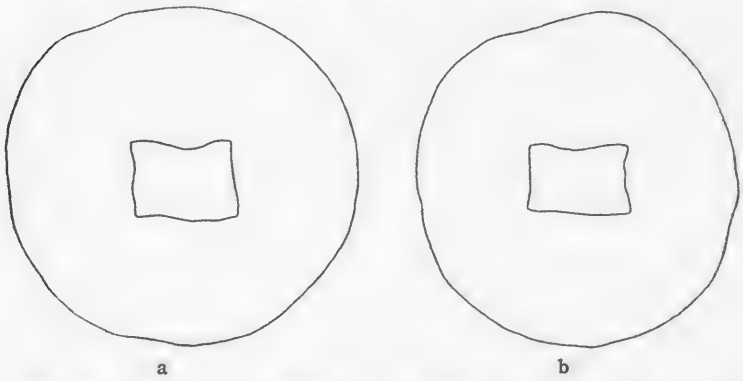
The only means of measuring the growth of a fragment of tissue accurately would be to measure the increase of its weight. Amounts of tissue large enough to be weighed can be easily cultivated *in vitro*, but as the cells cannot be separated from the fibrin of the medium, it is impossible to use this method. The enumeration of the cells contained in the medium would allow an accurate measurement of the increase in number, but connective tissue cells grow in the shape of a tissue, and it is impossible to count them. Therefore, the increase in mass of a fragment of tissue has to be ascertained by indirect meth-

ods. The only practical way was to estimate the increase in mass by the increase in area, the thickness being kept constant. When proper care is taken, the thickness of the new tissue does not vary and can, therefore, be disregarded. The measurement of the areas of new tissue can be made by two different techniques, either by measuring the width of the ring of fibroblasts surrounding the original fragment, or by measuring the area of the new growth. The dense growth of fibroblasts which surrounds a fragment of the old strain of connective tissue after 48 hours is neatly outlined on its outer and inner border. However, when the growth is very active, the inner border, that is the edge of the primitive fragment, becomes indistinct. Generally the side which corresponds in each piece of tissue to the central part of the fragment from which it was derived can be seen distinctly, because it is thicker and darker. It sometimes happens that the growth of the side corresponding to the central part of the mother fragment grows less actively than the three peripheral sides Only the width of the growth of symmetrical sides should be compared in both pieces. The measurements are made with an ocular micrometer. According to the activity of the tissue and the culture medium the width of the ring of new tissue varies between 0.5 and 8.00. The, old strain, when it is in active condition, produces in 48 hours a ring 7 or 8 divisions in width; that is, about 1.4 or 1.6 mm. This measurement does not take into account the increase in size of the original fragment which always occurs when embryonic heart is used. Again, it does not give precise results when, in a very active strain of connective tissue, the limit of new growth and the original fragment after 48 hours have become indistinct. In the case of the old strain, growing at a moderate rate, when the edges of the old plasma remain clear, this method has a sufficient amount of accuracy and the great advantage of being very simple and rapid.

The measurement of the surface of the new growth gives more precise results, but must be made by a more elaborate technique. As soon as the cultures are completed, before the beginning of the growth, the tissues are examined. This shows whether the fragments are still the same size and thickness, whether the edges have been cut neatly, whether no fibrin remains around the tissue, whether there is no folding of the edges of the old plasma, and whether the coagula-

tion of the new medium has taken place in the proper way. If both cultures are not identical in all of these details, they should be rejected. If they are found satisfactory, they are placed in a projecting apparatus and the outline of the fragment is traced on a sheet of paper (Text-fig. 1). Care should be taken to prevent the cultures from being overheated while under the projecting apparatus, and to keep

TEXT-FIG. 1. Experiment 18539; Passage 1657. Areas of two fragments, derived from the same culture, 1 hour after transfer into fresh medium. Area a = 4.5 sq. cm. Area b = 4.1 sq. cm. Reduced about one-third off.



TEXT-FIG. 2. Experiment 18539; Passage 1657. Area of the two fragments shown in Text-fig. 1, 48 hours later. Area a = 56.25 sq. cm. Area b = 49.4 sq. cm. The relative increase of Area a is 11.5 and of Area b 11.2, and the difference is 2 per cent. Reduced about one-third off.

each one outside of the incubator about the same length of time. There is no difficulty either with fresh tissue or with the old strain in making these first drawings. The second drawing is made after 48 hours (Text-fig. 2). The original fragment is again traced and also the outline of the new growth. When the old strain is used, there is generally no difficulty in tracing the outline of the original

fragment, although the cut edges of the old plasma may be covered by so many cells that they have become indistinct. This always happens when heart tissue from a very young embryo is cultivated. The outer edge of the dense ring of fibroblasts can easily be traced. The scattered cells which may be located beyond the limits of the dense growth should be disregarded. If the thickness of the new tissue is irregular, or if the outer edge is irregular and indistinct, the culture should be rejected.

Afterwards, the area of the original fragment and the total area after 48 hours are measured with the planimeter and expressed in square centimeters. The total area minus the area of the original fragment represents the absolute increase of the fragment. This area divided by the area of the primitive fragment is the relative increase of the fragment. The relative increase of the larger fragment divided by the relative increase of the smaller fragment expresses the difference in growth.

III. Results Obtained with Two Fragments of the 9 Year Old Strain of Connective Tissue.

Two series of thirty-two experiments were made, the results of which are shown in Tables I and II. Table I is composed of experiments made with a technique previously described,[3] in which no special care was taken in the selection of the fragments of tissues. In seventeen experiments, the differences between the growth of the two fragments are less than 10 per cent. In nine, it varies between 10 and 20 per cent; in four, between 20 and 30 per cent, and in the last two, the difference in one is 33 per cent, and in the other 51 per cent.

The results of the thirty-two experiments contained in Table II show the value of the improved technique. In twenty-five experiments, the difference in growth is less than 6 per cent; in seven, it varies between 6 and 7 per cent.

These results mean that this technique can be used profitable when the factors acting on the rate of growth are supposed to increase it by more than 10 per cent.

[3] Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 531–534.

TABLE I.

No. of experiment.	Culture No.	Date.	Passage No.; old strain.	Surface area of fragment	Surface area of growth.	Relative increase.	Ratio.	Difference.
		1920		*sq. cm.*	*sq. cm.*			
1	16691-1	June 5	1565	2.3	40.7	17.7	1.0	0.0
	16691-2			2.5	44.1	17.64		
2	16694-1	" 5	1565	2.0	30.3	15.5	1.09	0.09
	16694-2			2.2	36.4	16.54		
3	16695-1	" 5	1565	4.7	66.0	14.04	1.12	0.12
	16695-2			5.0	77.3	15.46		
4	17029-1	July 8	1581	3.0	71.9	23.96	1.12	0.12
	17029-2			2.6	55.4	21.3		
5	17030-1	" 8	1581	4.2	91.5	21.78	1.08	0.08
	17030-2			3.4	68.8	20.23		
6	17031-1	" 8	1581	3.1	53.1	17.13	1.10	0.10
	17031-2			3.5	66.5	19.0		
7	17032-1	" 8	1581	3.7	97.0	26.21	1.26	0.26
	17032-2			4.0	80.5	20.12		
8	17059-1	" 12	1583	2.1	34.0	16.2	1.04	0.04
	17059-2			2.0	31.3	15.5		
9	17062-1	" 12	1583	2.1	42.6	20.2	1.04	0.04
	17062-2			2.1	40.9	19.47		
10	17066-1	" 12	1583	3.6	38.6	10.72	1.09	0.09
	17066-2			4.0	39.0	9.75		
11	17075-1	" 12	1582	1.6	39.0	24.47	1.51	0.51
	17075-2			2.0	30.6	15.3		
12	17140-1	" 20	1587	5.8	46.7	8.05	1.07	0.07
	17140-2			5.0	43.3	8.66		
13	17143-1	" 20	1588	3.2	49.1	15.34	1.20	0.20
	17143-2			3.4	43.2	12.7		
14	17145-1	" 20	1588	3.5	36.5	10.45	1.33	0.33
	17145-2			3.7	29.1	7.86		
15	17146-1	" 20	1588	2.5	34.2	13.68	1.15	0.15
	17146-2			2.8	44.3	15.82		
16	17148-1	" 20	1586	3.1	48.3	15.58	1.28	0.28
	17148-2			3.5	42.6	12.17		

TABLE I—*Concluded*

No. of experiment.	Culture No.	Date.	Passage No.; old strain.	Surface area of fragment.	Surface area of growth.	Relative increase.	Ratio.	Difference.
		1920		*sq. cm.*	*sq. cm.*			
17	17200-1	July 26	1591	4.0	49.7	12.42	1.19	0.19
	17200-2			3.5	55.6	14.88		
18	17201-1	" 26	1591	3.5	55.6	14.88	1.06	0.06
	17201-2			3.9	58.7	14.05		
19	17202-1	" 26	1589	3.1	66.3	21.38	1.15	0.15
	17202-2			3.5	64.7	18.48		
20	17203-1	" 26	1589	4.7	63.8	13.57	1.02	0.02
	17203-2			5.2	72.1	13.86		
21	17207-1	" 26	1589	5.5	75.3	13.67	1.19	0.19
	17207-2			6.3	72.7	11.54		
22	17580-1	Sept. 13	1613	6.6	92.2	13.9	1.25	0.25
	17580-2			4.5	78.8	17.4		
23	17586-1	" 13	1613	5.4	67.9	12.5	1.05	0.05
	17586-2			5.4	64.2	11.9		
24	17589-1	" 13	1613	4.7	78.9	16.7	1.13	0.13
	17589-2			5.2	76.7	14.7		
25	17591-1	" 13	1613	4.5	42.1	9.3	1.02	0.02
	17591-2			5.3	50.4	9.5		
26	17592-1	" 13	1613	5.5	81.8	14.8	1.02	0.02
	17592-2			5.8	83.6	14.4		
27	18120-1	Nov. 8	1643	2.5	42.5	17.0	1.08	0.08
	18120-2			2.6	40.8	15.7		
28	18121-1	" 8	1643	4.5	52.0	11.6	1.01	0.01
	18121-2			4.6	51.9	11.3		
29	18122-1	" 8	1643	3.0	43.0	16.3	1.18	0.18
	18122-2			3.2	44.2	13.8		
30	18282-1	" 20	1648	4.9	59.1	12.1	1.03	0.03
	18282-2			5.0	62.6	12.5		
31	18285-1	" 20	1468	6.9	52.1	7.55	1.09	0.09
	18285-2			6.1	49.9	8.3		
32	18289-1	" 20	1648	6.4	64.6	10.1	1.0	0.09
	18289-2			7.0	70.0	10.0		

TABLE II.

No. of experiment.	Culture No.	Date.	Passage No.; old strain.	Surface area of fragment.	Surface area of growth.	Relative increase.	Ratio.	Difference.
		1920		*sq. cm.*	*sq. cm.*			
1	18334-1	Nov. 24	1650	4.2	50.3	12.0	1.06	0.06
	18334-2			3.0	38.3	12.73		
2	18337-1	" 24	1648	4.4	56.2	12.8	1.0	0.0
	18337-2			4.3	55.2	12.9		
3	18340-1	" 24	1650	6.2	47.6	7.7	1.07	0.07
	18340-2			5.4	44.9	8.3		
4	18416-1	" 29	1653	3.5	49.9	14.2	1.05	0.05
	18416-2			3.7	48.6	13.5		
5	18417-1	" 29	1653	4.0	66.9	15.7	1.05	0.05
	18417-2			4.0	68.2	16.0		
6	18419-1	" 29	1653	2.0	30.3	15.5	1.06	0.06
	18419-2			2.2	36.4	16.5		
7	18449-1	Dec. 1	1654	3.0	60.0	20.0	1.04	0.04
	18449-2			3.2	69.9	20.84		
8	18462-1	" 1	1654	2.8	30.24	10.8	1.02	0.02
	18462-2			3.0	33.1	11.0		
9	18463-1	" 1	1654	3.3	50.2	15.5	1.02	0.02
	18463-2			3.9	61.6	15.8		
10	18516-1	" 4	1654	3.9	49.7	12.74	1.02	0.02
	18516-2			2.9	37.7	13.0		
11	18518-1	" 4	1655	3.7	43.3	11.7	1.05	0.05
	18518-2			3.9	44.8	11.43		
12	18522-1	" 4	1656	4.1	53.2	12.97	1.07	0.07
	18522-2			4.1	57.3	13.97		
13	18534-1	" 6	1657	1.8	34.6	19.2	1.02	0.02
	18534-2			1.7	33.3	19.6		
14	18535-1	" 6	1657	3.7	39.3	10.5	1.03	0.03
	18535-2			3.8	42.1	10.01		
15	18539-1	" 6	1657	4.5	51.75	11.5	1.02	0.02
	18539-2			4.1	45.3	11.2		
16	18575-1	" 8	1658	2.3	40.7	17.7	1.0	0.0
	18575-2			2.5	44.1	17.64		

TABLE II—*Concluded.*

No. of experiment.	Culture No.	Date.	Passage No.; old strain.	Surface area of fragment.	Surface area of growth.	Relative increase.	Ratio.	Difference.
		1920		*sq. cm.*	*sq. cm.*			
17	18579-1	Dec. 8	1658	5.0	60.1	12.0	1.01	0.01
	18579-2			5.0	61.2	12.2		
18	18580-1	" 8	1658	4.3	50.7	11.66	1.0	0.0
	18580-2			4.2	49.2	11.66		
19	18621-1	" 11	1659	5.2	63.6	12.21	1.02	0.02
	18621-2			5.3	65.5	12.17		
20	18625-1	" 11	1660	3.5	73.0	20.85	1.01	0.01
	18625-2			4.2	77.1	21.0		
21	18628-1	" 11	1660	4.7	64.0	14.01	1.01	0.01
	18628-2			5.0	71.5	14.3		
22	18768-1	" 20	1664	5.1	67.8	13.3	1.06	0.06
	18768-2			5.0	70.1	14.02		
23	18773-1	" 20	1665	4.7	68.3	14.47	1.05	0.05
	18773-2			4.9	67.7	13.69		
24	18777-1	" 20	1667	5.1	77.6	16.1	1.07	0.07
	18777-2			5.0	86.4	17.28		
25	18904-1	" 26	1668	2.5	42.8	17.12	1.05	0.05
	18904-2			2.5	40.8	16.32		
26	18908-1	" 26	1667	4.6	76.8	16.68	1.0	0.0
	18908-2			4.7	73.4	16.68		
27	18909-1	" 26	1667	3.7	68.8	18.52	1.04	0.04
	18909-2			5.0	88.1	17.62		
		1921						
28	19015-1	Jan. 1	1669	4.4	25.0	5.68	1.02	0.02
	19015-2			4.1	23.8	5.8		
29	19024-1	" 1	1670	3.3	62.2	18.88	1.03	0.03
	19024-2			3.2	62.7	19.6		
30	19309-1	" 24	1682	3.5	16.06	3.59	1.03	0.03
	19309-2			4.6	20.5	3.47		
31	19320-1	" 25	1682	3.7	30.7	8.56	1.07	0.07
	19320-2			4.6	36.5	7.96		
32	19495-1	Feb. 7	1683	3.7	32.1	8.67	1.0	0.0
	19495-2			4.4	38.1	8.65		

CONCLUSIONS.

1. A technique has been developed by which two fragments of tissue, put in identical media, can grow at almost the same rate.

2. A method of measuring the growing tissue is described.

3. The differences observed in the rate of growth of two fragments of the 9 year old strain of connective tissue, cultivated in identical media and measured according to this technique, are less than 10 per cent.

STUDIES ON PNEUMOCOCCUS IMMUNITY.

I. Active Immunization of Monkeys against Pneumococcus Type I Pneumonia with Pneumococcus Type I Vaccine.*

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In an article recently published on the results of prophylactic vaccination against pneumococcus pneumonia in monkeys, Cecil and Blake[1] found that subcutaneous inoculation with Pneumococcus Type I vaccine in doses comparable to those employed in man did not protect monkeys against subsequent attacks of Pneumococcus Type I pneumonia, either spontaneous or experimental. Vaccination did, however, modify the course of the disease. Invasion of the blood stream by the pneumococcus was usually slight or absent in vaccinated animals, and the proportion of recoveries was considerably higher for vaccinated than for unvaccinated monkeys. In the experiments of Cecil and Blake, comparatively small doses of vaccine were used. In view of the failure, however, of small doses of vaccine to give complete protection against pneumonia, it seemed desirable to determine the effect of large doses of pneumococcus vaccine.

In the following experiments, three species of monkeys have been employed. *Macacus rhesus* was used in three of the experiments, *Macacus syrichtus* in one, and the *Cebus capucinus* in one. *Macacus rhesus* is less susceptible to pneumococcus infection than the other two species, and rarely if ever develops a true lobar pneumonia. The infection is more apt to be of the interstitial or confluent lobular type. The Philippine macaque (*Macacus syrichtus*) is the preferable animal for studying experimental pneumococcus pneumonia, as it develops a

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[1] Cecil, R. L., and Blake, F. G., *J. Exp. Med.*, 1920, xxxi, 519.

true lobar consolidation. At the time these experiments were carried out, however, it was not possible to obtain this species in any considerable number. *Cebus capucinus* occupies an intermediate position in respect to susceptibility to pneumococcus pneumonia. In some cases it may present a typical lobar consolidation, but, more often, this species, like the *rhesus*, develops only an interstitial or patchy consolidation, which is usually associated with a heavy pneumococcus septicemia.

Methods.

The vaccine employed for these experiments was prepared from a stock laboratory strain of Pneumococcus Type I. This organism was highly virulent, killing a mouse in quantities as small as 0.0000001 cc. of a 24 hour broth culture. The vaccine was prepared as follows: 18 to 24 hour glucose broth cultures were centrifuged, the sediment was washed with normal salt solution, and then resuspended in normal salt solution in such a concentration that 0.5 cc. equalled 20 billion pneumococci. The suspension was heated to 55° C. for 1 hour to kill the pneumococci, and 0.25 per cent tricresol added as a preservative. With one exception (Experiment 3), the same dosage of vaccine was employed in all the experiments. Three subcutaneous injections were given at intervals of 1 week, the first dose consisting of 20 billion pneumococci, the second of 40 billion, and the third of 60 billion. The injections were made in the abdominal wall, and caused very little local or general reaction.

The culture used for testing the immunity of the vaccinated monkeys was the same strain of Pneumococcus Type I from which the vaccine had been prepared. The method of producing experimental pneumonia in monkeys has been previously described by Blake and Cecil.[2] A small quantity of an 18-hour broth culture of pneumococcus is introduced with a Luer syringe through the skin into the trachea. In testing for resistance to pneumonia following the injection of pneumococcus vaccine, the intratracheal inoculations were made in most cases 2 to 3 weeks after the completion of vaccination. The dose of culture injected varied from 0.01 to 0.000001 cc. for each monkey. A further test of the immunity of each vaccinated monkey was made

[2] Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 403.

by withdrawing some blood just before intratracheal inoculation, and carrying out protection tests in mice with the serum of the monkey.

In most cases the monkeys died or were killed at the conclusion of the experiment. Complete autopsies were performed in each instance and microscopic sections studied from the various lobes of the lungs.

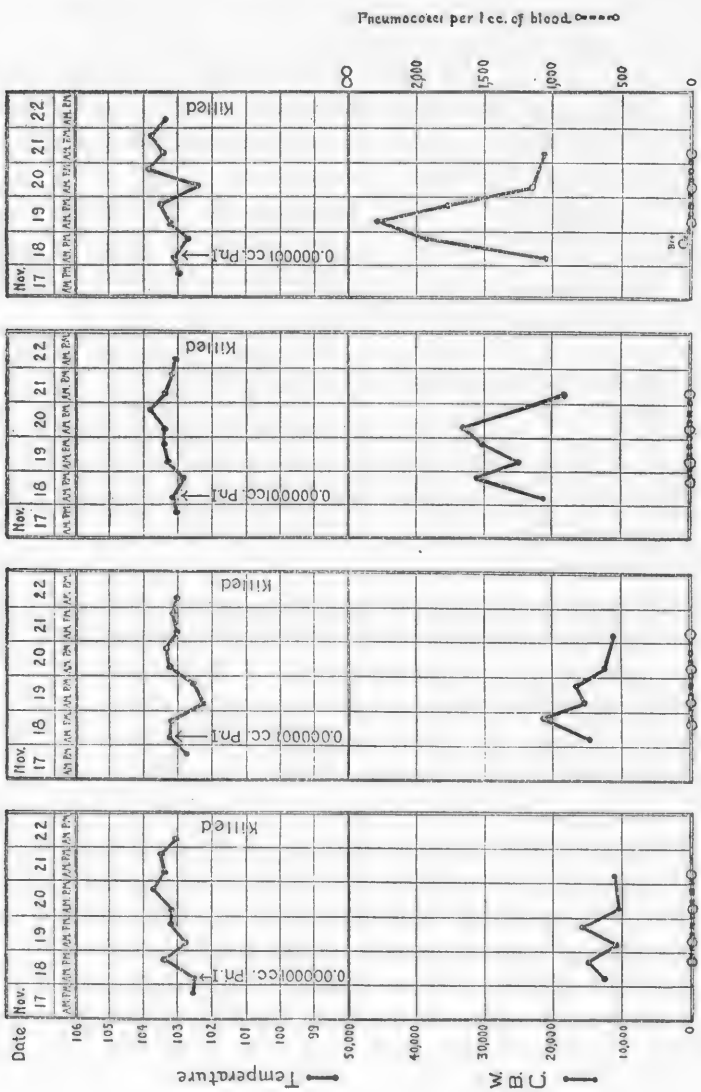
Active Immunity in Macacus rhesus Following Vaccination with Large Doses of Pneumococcus Type I Vaccine.

The first experiment with large doses of Pneumococcus Type I vaccine was carried out on *rhesus* monkeys.

Experiment 1.—Oct. 21, 1919. Three *Macacus rhesus* monkeys (Nos. 1 to 3) received each 20 billion (0.5 cc.) Pneumococcus Type I saline vaccine subcutaneously. Oct. 22. Mild local reactions. Oct. 28. Each monkey received 40 billion (1 cc.) Pneumococcus Type I vaccine subcutaneously. Nov. 4. Each monkey received 60 billion (1.5 cc.) Pneumococcus Type I vaccine subcutaneously. Nov. 18. 2 weeks after last injection of vaccine, each of the three monkeys received 0.000001 cc. of an 18hour broth culture of Pneumococcus Type I intratracheally. An unvaccinated control monkey (No. 12) received the same amount of culture intratracheally.

The results are shown in Text-fig. 1. The three vaccinated monkeys remained perfectly well, showed practically no change in temperature or leucocyte count, and had negative blood cultures throughout the period of observation. The control was sick for 3 days, developed a leucocytosis of 47,000, and had a transient pneumococcus septicemia. All four animals were killed on the 5th day following inoculation. The lungs in the vaccinated monkeys were entirely free from pneumonia. In the control a small patch of interstitial pneumonia was found in the left lower lobe. Cultures from the lungs and heart's blood were sterile in all the monkeys.

Protection Tests.—The serums from the three vaccinated monkeys were tested for specific protective bodies against Pneumococcus Type I. On November 18, just before intratracheal inoculation, the three vaccinated monkeys were bled for protection tests in mice. The serum of Monkey 1 developed a moderate amount of protective substance, protecting a mouse against 0.0000001 cc. of Pneumococcus Type I culture. The serum of Monkey 2 protected mice against 0.0001 cc. of Pneumococcus Type I culture. The serum of Monkey 3 showed practically no protective power.



TEXT-FIG. 1, *a* to *d*. Active immunity against Pneumococcus Type I pneumonia following vaccination with Pneumococcus Type I vaccine. (*a*), (*b*), and (*c*) Monkeys 1, 2, and 3; each received 120 billion Pneumococcus Type I vaccine subcutaneously. (*d*) Monkey 12; control. *Br.*+, Broth culture positive for Pneumococcus Type I.

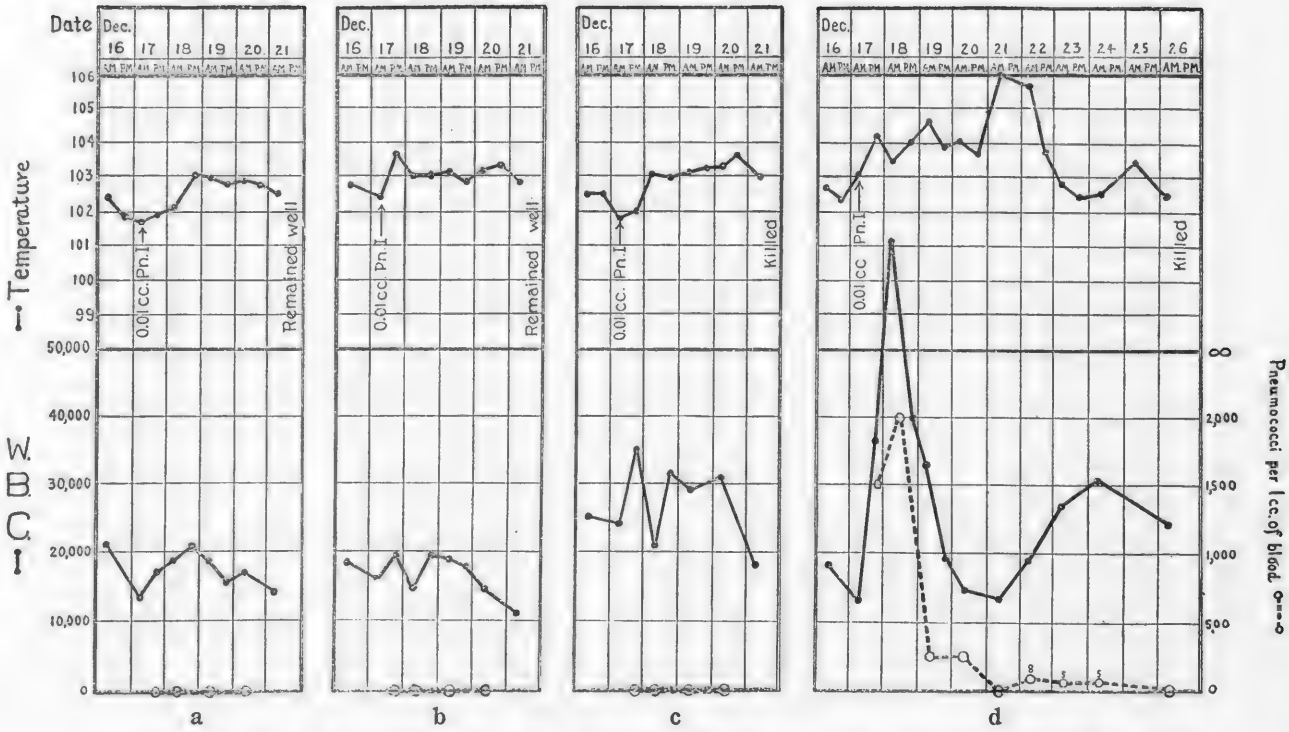
It is evident from this experiment that three large subcutaneous doses of Pneumococcus Type I vaccine conferred complete protection against pneumonia. The result, however, was unsatisfactory in one respect; the control developed only a small patch of interstitial pneumonia and was not seriously ill. The experiment was therefore repeated on the same species with larger infecting doses of Pneumococcus Type I culture.

Experiment 2.—Nov. 24, 1919. Three *Macacus rhesus* monkeys (Nos. 13 to 15) received each 20 billion (0.5 cc.) Pneumococcus Type I saline vaccine subcutaneously. Nov. 27. Small nodules at site of vaccine injection. Dec. 1. Each monkey received 40 billion (1 cc.) Pneumococcus Type I vaccine subcutaneously. Dec. 6. Small nodules at site of previous inoculation. In Monkey 13 the nodule has broken down and is discharging sterile pus. Dec. 7. Each monkey received 60 billion (1.5 cc.) Pneumococcus Type I vaccine subcutaneously. Dec. 17. Each monkey received 0.01 cc. of an 18 hour broth culture of Pneumococcus Type I intratracheally, and an unvaccinated control monkey (No. 21) received the same amount (0.01 cc.) of culture intratracheally.

The results are shown in Text-fig. 2. The three vaccinated monkeys remained lively and well, and showed no noteworthy changes in temperature or leucocyte count. The blood remained sterile in the three vaccinated monkeys. The control monkey developed the signs and symptoms of pneumonia and recovered by crisis on the 6th day. One of the vaccinated monkeys (No. 15) and the control monkey were killed. The vaccinated monkey showed normal lungs. The control showed a resolving pneumonia of the interstitial type involving the right upper, middle, and lower lobes and the left upper lobe. Cultures taken at autopsy from the lungs and heart's blood of both monkeys were sterile.

Protection Tests.—Blood was taken from the veins of the three vaccinated monkeys just previous to the intratracheal inoculations for the purpose of determining the protective power of their serums. In spite of the high degree of active immunity conferred by the vaccine, none of the serums showed any protection for mice.

This experiment corroborated the findings in Experiment 1 and indicates that three large subcutaneous injections of Pneumococcus Type I vaccine confer complete protection against the homologous type of pneumonia.



TEXT-FIG. 2, *a* to *d*. Active immunity against Pneumococcus Type I pneumonia following vaccination with Pneumococcus Type I vaccine. (*a*), (*b*), and (*c*) Monkeys 13, 14, and 15; each received 120 billion Pneumococcus Type I vaccine subcutaneously. (*d*) Monkey 21; control.

It should be noted that in these experiments, as in the previously reported experiments of Cecil and Blake, the amount of protective substance in the serum of vaccinated monkeys varies widely with different individuals and apparently bears no close relation to local immunity in the lungs, a phenomenon which appears to be fairly constant following vaccination.

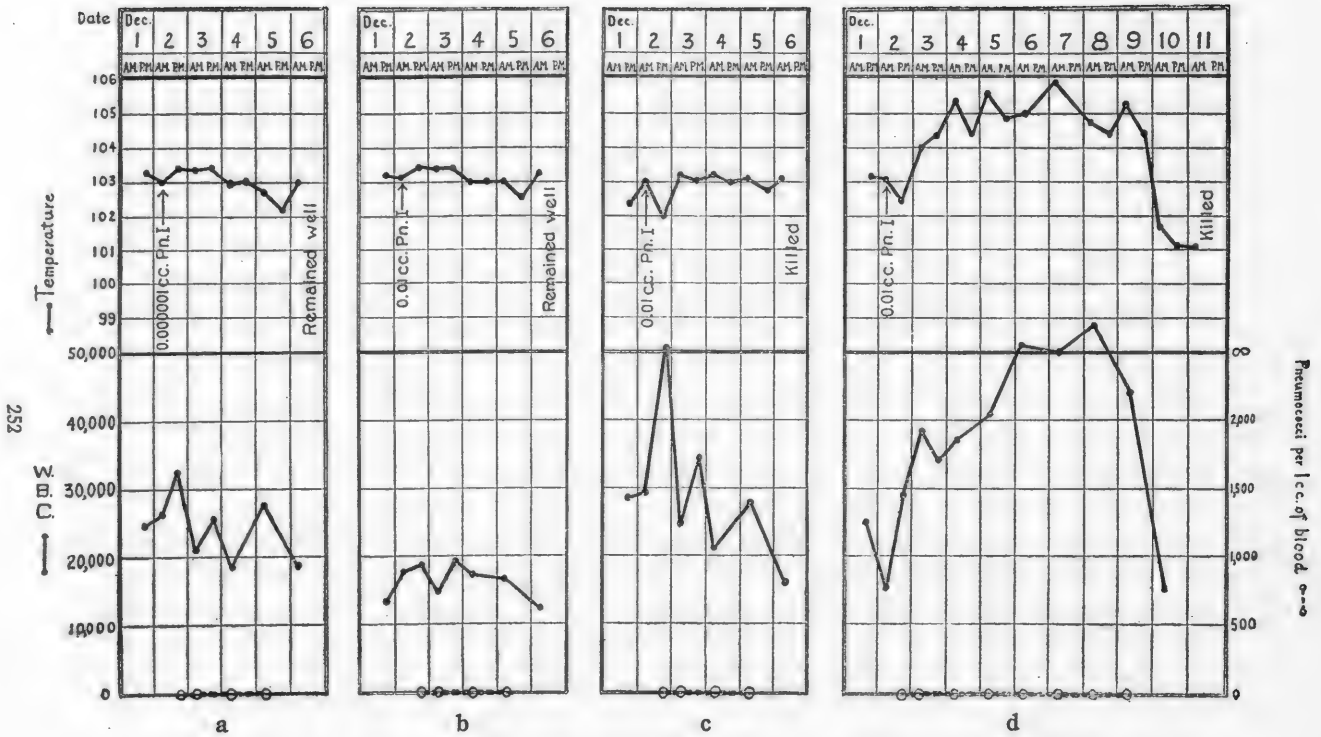
Active Immunity Following Intravenous Vaccination with Small Doses of Pneumococcus Type I Vaccine.

In order to determine the effect of intravenous injections of pneumococcus vaccine, three *rhesus* monkeys were injected intravenously with small doses of Pneumococcus Type I vaccine and their immunity was tested by intratracheal inoculation of virulent pneumococci as in the preceding experiments.

Experiment 3.—Oct. 27, 1919. Three *Macacus rhesus* monkeys (Nos. 4, 5, and 10) received each 300 million Pneumococcus Type I saline vaccine intravenously. Nov. 3. Each monkey received 400 million Pneumococcus Type I vaccine intravenously. Nov. 4. Monkeys lively and well. Nov. 10. Each monkey received 800 million Pneumococcus Type I vaccine intravenously. Nov 11. Animals lively and well. Nov. 17. Each monkey received 1,600 million Pneumococcus Type I vaccine intravenously. Dec. 2. Each monkey received 0.01 cc. of an 18 hour broth culture of a Pneumococcus Type I intratracheally. An unvaccinated control monkey (No. 17) received the same amount (0.01 cc.) of culture intratracheally.

The results are shown in Text-fig. 3. The three vaccinated monkeys remained lively and well. Monkey 10 had a sharp leucocyte reaction but showed no other signs of infection. The control monkey became ill and showed symptoms of pneumonia. One of the vaccinated monkeys (No. 10) and the control monkey (No. 17) were killed. Monkey 10 showed an acute bronchitis but no pneumonia. The control monkey presented a complete consolidation of the right upper lobe, and, microscopically, resolving pneumonia of the interstitial type was demonstrated. Cultures taken at autopsy from the lungs and heart's blood of these two monkeys were in both cases sterile.

Protection Tests.—The vaccinated monkeys were bled previous to the intratracheal inoculations in order to test the protective power of their serums. Monkey 10 showed a high degree of protective



TEXT-FIG. 3, *a* to *d*. Active immunity against Pneumococcus Type I pneumonia following vaccination with Pneumococcus Type I vaccine. (*a*), (*b*), and (*c*) Monkeys 4, 5, and 10; each received 3 billion Pneumococcus Type I vaccine intravenously. (*d*) Monkey 17; control.

substance in its serum which protected mice against 0.0001 cc. of Pneumococcus Type I culture; in Monkey 4 there was a small amount of protective substance, the mouse receiving 0.0000001 cc. of Pneumococcus Type I surviving. The serum of Monkey 5 showed no protective power at all.

This experiment demonstrates that small doses of Pneumococcus Type I vaccine injected intravenously confer an immunity equivalent to that obtained by large doses of pneumococcus vaccine administered subcutaneously. This is in harmony with the observations of many immunologists that immunity can be obtained more readily by intravenous than by subcutaneous inoculations.

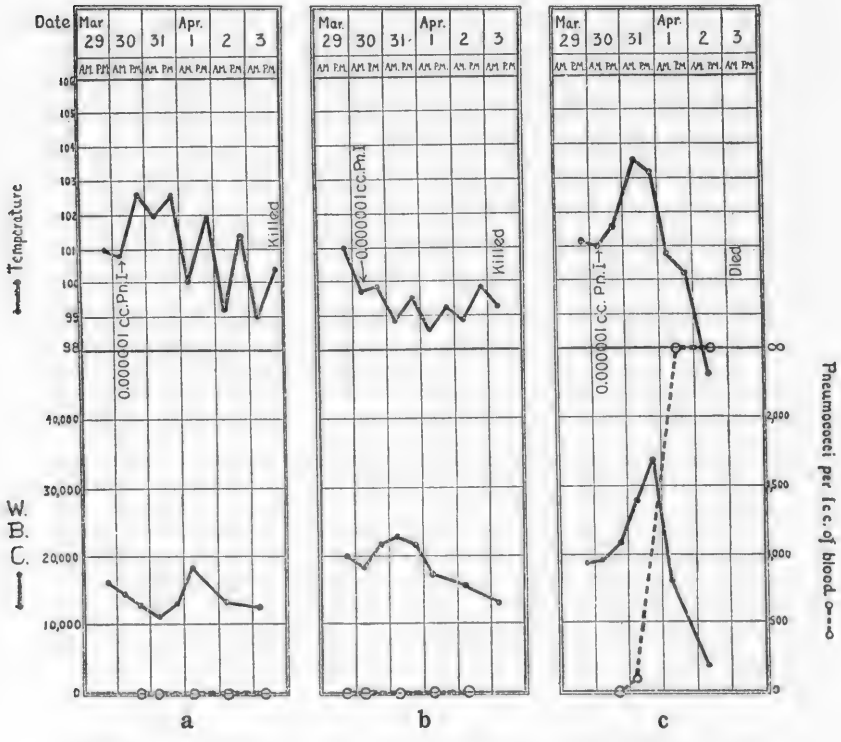
Active Immunity in Philippine Monkeys Following Vaccination with Large Doses of Pneumococcus Type I Vaccine.

In view of the fact that most of the experiments carried out by Cecil and Blake in their study of prophylactic vaccination against pneumonia in monkeys were performed on *Macacus syrichtus*, it seemed desirable to control the result obtained in the above described experiments by an experiment on Philippine monkeys.

Experiment 4.—Feb. 27, 1920. Two Philippine monkeys (Nos. 34 and 35) received each 20 billion (0.5 cc.) Pneumococcus Type I saline vaccine subcutaneously. Feb. 28. Monkeys lively and well. Mar. 5. Each monkey received 40 billion (1 cc.) Pneumococcus Type I vaccine subcutaneously. Mar. 12. Each monkey received 60 billion (1.5 cc.) Pneumococcus Type I vaccine subcutaneously. Mar. 30. Each monkey received 0.000001 cc. of an 18 hour broth culture of Pneumococcus Type I intratracheally. An unvaccinated control monkey (No. 36) received the same amount (0.000001 cc.) of culture intratracheally.

The results are shown in Text-fig. 4. The two vaccinated monkeys remained lively and well. Their blood remained free from bacteria and the temperature and leucocytes showed no significant variation. The control monkey developed pneumonia and an overwhelming pneumococcus septicemia and died on the 5th day of the disease. The two vaccinated monkeys were killed on the 5th day following inoculation. Monkey 34 showed several clusters of small gray tubercles but there was no pneumonic consolidation. The presence of tuberculosis in this monkey explains the morning and evening varia-

tions in temperature (Text-fig. 4). Monkey 35 also showed a patch of encapsulated tuberculosis in the left upper lobe but there was no fresh consolidation. The control monkey showed an early pneumococcus pneumonia (stage of engorgement), involving the right and



TEXT-FIG. 4, *a* to *c*. Active immunity against Pneumococcus Type I pneumonia in Philippine monkeys following vaccination with Pneumococcus Type I vaccine. (*a*) and (*b*) Monkeys 34 and 35; each received 120 billion Pneumococcus Type I vaccine subcutaneously. (*c*) Monkey 36; control.

left lower lobes. Cultures at autopsy from the lungs and heart's blood of the vaccinated monkey were sterile. Films from the tuberculous foci showed tubercle bacilli. Cultures from the lungs and heart's blood of the control monkey gave Pneumococcus Type I.

Protection Tests.—The serums of the two vaccinated monkeys were tested for protective substances. Monkey 34 showed a definite amount of protective substance in its serum, the mice receiving 0.0000001 and 0.00001 cc. of Pneumococcus Type I both surviving. With the serum from Monkey 35 only one mouse survived—the one receiving 0.0000001 cc. of culture.

This experiment shows that even in the case of the Philippine macaque, a monkey peculiarly susceptible to pneumonia, an adequate immunity against Pneumococcus Type I pneumonia may be obtained by the administration of three large subcutaneous injections of Pneumococcus Type I vaccine.

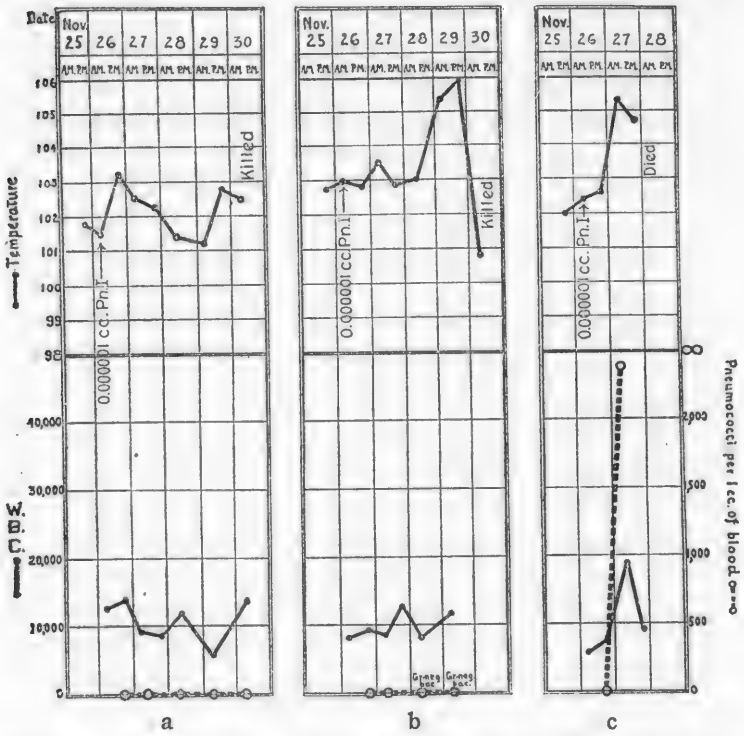
Active Immunity in Cebus capucinus Following Vaccination with Three Large Doses of Pneumococcus Type I Vaccine.

A final test of Pneumococcus Type I vaccine was carried out on the small South American ringtail. This species was being used for other pneumococcus experiments and it seemed desirable to determine whether an efficient immunity against pneumococcus pneumonia could be obtained in this species by means of three subcutaneous inoculations of pneumococcus vaccine.

Experiment 5.—Oct. 29, 1920. Two *Cebus capucinus* monkeys (Nos. 82 and 83) received each 20 billion (0.5 cc.) Pneumococcus Type I saline vaccine subcutaneously. Nov. 3. Small nodules at site of previous inoculation. Nov. 4. Each monkey received 40 billion (1 cc.) Pneumococcus Type I vaccine subcutaneously. Nov. 11. Each monkey received 60 billion (1.5 cc.) Pneumococcus Type I saline vaccine subcutaneously. Nov. 26. Each monkey received 0.0001 cc. of a 24 hour broth culture of Pneumococcus Type I intratracheally. An unvaccinated control monkey (No. 86) received the same amount (0.0001 cc.) of culture intratracheally.

The results are shown in Text-fig. 5. Monkey 82 remained lively and well and showed no signs of infection. Monkey 83 remained well until the 4th day following inoculation, when he unfortunately contracted distemper. An epidemic of this disease had broken out among the stock monkeys and, in spite of the fact that the subjects under experiment were isolated in separate cages, Monkey 83 contracted the disease and was quite ill when he was killed on the 5th day following inoculation. Autopsy showed lobar pneumonia of the

right middle and left lower lobes, and *Bacillus bronchisepticus* was obtained in pure culture from the lungs. There was no evidence of a pneumococcus infection at any time during the experiment. Monkey 82 was also killed on the 5th day following inoculation and the



TEXT-FIG. 5, *a* to *c*. Active immunity against Pneumococcus Type I pneumonia in Capuchin monkeys following vaccination with Pneumococcus Type I vaccine. (*a*) and (*b*) Monkeys 82 and 83; each received 120 billion Pneumococcus Type I vaccine subcutaneously. (*c*) Monkey 86; control.

lungs were found free from consolidation. Monkey 86, the control, died with an overwhelming pneumococcus septicemia on the 3d day following inoculation. At autopsy the lungs showed engorgement and patches of incipient pneumonia in the right middle and left lower

lobes, and cultures taken from the lungs and from the heart's blood showed a pure growth of Pneumococcus Type I.

Protection Tests.—Blood was taken from the two vaccinated monkeys just before the intratracheal inoculations and was tested on mice for the presence of specific protective substance against Pneumococcus Type I. Contrary to the usual experience, no protective bodies could be demonstrated in either of the vaccinated monkeys.

This experiment corroborates the results obtained in the previous experiments and shows that in Capuchin monkeys three large subcutaneous injections of pneumococcus Type I vaccine confer a high degree of active immunity against the homologous type of pneumonia.

DISCUSSION.

The experiments reported in this paper establish a fact which has at least a theoretical importance. They demonstrate that monkeys can be completely protected against Pneumococcus Type I pneumonia by means of three subcutaneous injections of Pneumococcus Type I vaccine, provided sufficiently large doses are administered. The total dosage of vaccine employed in these studies averages about ten times as large as that used by Cecil and Blake in their unsuccessful attempts to vaccinate monkeys against pneumonia. It may be argued that the dosage employed in the present study could not be made use of in man without exciting severe local and general reactions. It is doubtful, however, whether such large doses would be necessary in the case of man. The average, healthy human being probably possesses more resistance to pneumonia than the average monkey by reason of long continued exposure to pneumococcus infection. Furthermore, when monkeys are infected artificially by intratracheal inoculation, a large number of pneumococci (400 to 1,000,000) attack the organism at once, whereas, in spontaneous infection in man, the number of pneumococci attacking at the onset of pneumonia is probably very small. It would be desirable, however, to administer large doses of pneumococcus vaccine to man if the toxic element in the vaccine could be removed without injury to the antigenic element.

The results of the protection tests carried out with the serum of the vaccinated monkeys are in harmony with the results reported in the studies of Cecil and Blake. Protective substances may or may

not be present in the serum of vaccinated monkeys and appear to play little or no part in active immunity to pneumococcus infection in the lung itself.

The intravenous administration of pneumococcus vaccine induces a higher immunity than subcutaneous injection. The intravenous method however would be much more time-consuming and this would be an important consideration where large groups of individuals were to be vaccinated.

CONCLUSIONS.

1. The subcutaneous inoculation of monkeys with three large doses of Pneumococcus Type I vaccine confers on them a complete immunity against experimental Pneumococcus Type I pneumonia.

2. The intravenous inoculation of small doses of Pneumococcus Type I vaccine also confers complete immunity against the homologous type of pneumonia.

3. Specific protective bodies may or may not be present in the serum of monkeys vaccinated against Pneumococcus Type I. There appears to be no intimate relation between active immunity against pneumonia and the presence or absence of protective substances in the serum of the vaccinated animal.

DATA ON THE DEVELOPMENT OF HETERAKIS PAPILLOSA IN THE FOWL.

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PLATE 18.

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INTRODUCTION.

The demonstration that the round worm *Heterakis papillosa* inhabiting the ceca of chickens, turkeys, and certain other birds is a factor in the production of the disease popularly known as blackhead has emphasized the importance of further knowledge of the biology of this worm. Birds other than chickens and turkeys in which it has been reported are the peafowl, guinea fowl, various pheasants, partridge, grouse, quail, bustard, and the domestic duck and goose. Experimental work in the production of blackhead by feeding ova of this parasite had already indicated that its development was in all probability direct. The work presented in this paper was undertaken to determine the course of its life cycle within the host. Artificial incubation and brooding make it possible to rear young birds away from the environment of adults. Thus the problem of control is much simplified and resolves itself largely into one of determining how long ova will remain viable in soil. The danger of wild hosts introducing the parasite will vary with localities. In the locality in which our work has been done this has not proved to be a serious factor, but nevertheless it is one that must be taken into consideration.

Railliet and Lucet[1] and Ackert[2] succeeded in infesting chickens by feeding them developed eggs. The latter author also succeeded in infesting chickens by feeding dung earthworms (*Helodrilus gieseleri*

[1] Railliet, A., and Lucet, A., *Bull. Soc. zool. France*, 1892, xvii, 117.

[2] Ackert, J. E., *Science*, 1917, xlvi, 394.

hempeli Smith) taken from a poultry yard. He interpreted the result as indicating that ova had adhered to the worms or were present in the alimentary tract, but did not believe the evidence excluded the possibility that the earthworm might act in some way as an intermediate host.

Development Outside the Host.

The fully formed ova are deposited by the female worm in an unsegmented condition in the ceca. They are elliptical in shape and measure 74 to 78 microns long and 41 microns broad. The shell is thick, resistant, and provides a market protection against external conditions. It is fairly uniform in thickness and in a number of eggs ranged from 3.3. to 3.7 microns. It consists of two layers, the inner one frequently of a pale blue color and having a thickness of half or less of that of the outer layer. Between the two layers at one pole is a minute, clear, lenticular body, the significance of which has not been determined. The ova pass out with the cecal contents and undergo development on the ground. The embryo when fully grown lies coiled within the shell and as a rule shows very little tendency to move. The ends of the body are clear and the middle region is marked by a broad column of minute, highly refractive granules. Except in rare instances, to be considered accidental, the developed ova do not hatch until they reach the alimentary tract of a host.

Experiments have been undertaken to determine the incubation period of the eggs, the longevity of the contained embryos, and the resistance of developed and undeveloped eggs to low temperatures and desiccation. These have been conducted in some instances under conditions quite different from those found on the soil, but they serve to give an approximate guide to what takes place in nature.

In observations on the development and resistance of ova both solid and fluid media were used. In the former, ovigerous females were cut up and spread over a layer of salt solution agar consisting of 0.5 per cent salt and 2 per cent agar in Petri dishes. In the latter, worms were cut up and placed in a shallow layer of physiological salt solution. Evaporation was compensated for by adding distilled water. The agar simply provided a moist substratum, with moisture and oxygen conditions somewhat different from those in salt solution cultures.

Incubation.—An agar and a salt solution culture were placed in a refrigerator, the temperature of which taken on 7 days ranged from 2.5 –8°C. For a period of 24 days the ova failed to undergo development but developed when removed to room temperature. At the same time two similar cultures were placed in a cool room. The temperature was rather uniform and considerably below that of the laboratory rooms. Taken on 8 days it fluctuated between 11.5 and 13.5°C. Development proceeded slowly and it required 38 days for embryos to become completely formed. Eggs on agar and in salt solution developed at the same rate. These observations indicate that the minimum temperature at which ova develop lies somewhere between 8° and 11.5 –13.5°C. The slow rate of development at the latter temperatures indicates that they are not far removed from the critical point.

Sets of agar and salt solution cultures were held at room temperature. There was no difference in the rate of development in the two types of cultures. Cultures held at a temperature which taken on 6 days ranged from 19–23.2° C. showed fully developed eggs in 8 days and by the 11th day all had developed. Cultures kept in a somewhat warmer room, in which the temperature taken on 6 days ranged from 22–25.6°C., were developed on the 8th day. In a salt solution culture, in which the temperature observed on 2 days was 18° and 26°C., development was complete in 9 days. Another culture, in which the temperature noted on four occasions ranged from 20–29°C., developed in 7 to 12 days. The development of the ova at room temperature ranging approximately from 18–29°C. therefore required 7 to 12 days.

Resistance of Ova to Low Temperature.—To determine the resistance of undeveloped ova to freezing, a salt solution culture was held for nearly 4 days in a refrigerator at a temperature of freezing. When removed to a warm room these eggs developed and produced normal embryos.

An agar and a salt solution culture containing embryonated eggs were placed outdoors in an unheated building for 7 days, from February 27 to March 5, 1920. The maximum and minimum temperature for the period ranged from 5–62°F. The eggs after being removed to a warm room for examination appeared normal and embryos were observed that showed movement.

Desiccation.—To study the influence of desiccation on developed and undeveloped eggs, ova were placed on the bottom of an empty

Petri dish and exposed to the atmosphere of a room. From time to time some were removed and placed in salt solution to determine their viability.

Undeveloped ova dried for a period of 16 days during October, 1920, were viable but at the next observation at the end of 41 days were no longer capable of development. At about the same time developed eggs were subjected to desiccation in a similar manner. At the end of 18 days many embryos were alive although a certain percentage had perished. At the next observation on the 49th day all were dead. In another test during October embryos were viable on the 8th day, but on the 10th day appeared to be dead.

Longevity of Embryos.—The embryo within the egg has a remarkable longevity. The thickness of the egg shell offers a protection against evaporation and unfavorable conditions.

In two agar cultures made January 23, 1920, and kept at room temperature embryos were observed to be alive as late as July 2 and September 20, respectively. After these dates no observations were made.

In two salt solution cultures, made at the same time as the above and held under identical conditions, on January 25, 1921, after a period of 12 months, although some embryos had perished, most of them appeared normal and movement was observed in certain instances.

A determination of longevity was made under conditions more nearly approaching those in nature. Salt solution cultures were made September 26 and kept at room temperature. At the end of 12 days (October 8), when the embryos were fully formed, the contents of each were transferred to soil in a small flower pot and mixed up with the surface layer. These pots were embedded part way in a pan containing soil and placed outdoors in a cage of wire netting. At intervals the pots were taken to the laboratory, the ova removed and examined with regard to the state of the embryos. It was found that they were still alive after 8 months.[3]

[3] In a field experiment reported elsewhere (Graybill, H. W., *J. Exp. Med.*. 1921, xxxiii, 667), in which young turkeys were exposed to soil that had been unoccupied for a period of 5 months beginning in January, it was found that viable ova were still present.

Course of Larvæ Within the Host.

Young chickens were fed the developed ova of *Heterakis* and killed at intervals to determine the location of the larvæ and observe their state of development. The breeds used were White Leghorn, Rhode Island Red, and Plymouth Rock. When introduced into the experiment they were 32 days old. Some controls from the same stock were provided. The cultures were made in Petri dishes by cutting up female worms, collected from chickens, in a shallow layer of physiological salt solution. They were 14 and 27 days old when fed.

The chickens had been reared in incubators and brooders and during the course of the experiments were kept indoors and protected from infestation with *Heterakis*.

The results of the examination of the chickens are given in Table I. The presence or absence of worms in the various situations is indicated by means of positive and negative signs. Unless otherwise stated the method of searching for larvæ consisted in mounting one or more samples of mucus, or contents of regions of the alimentary tract, or scrapings from an organ, and examining the same under the microscope, using a mechanical stage to cover the whole mount. The method used in examining completely any region of the alimentary tract consisted in slitting it open in a mason jar containing water. The jar was then closed and shaken thoroughly to dislodge contents from mucosa and bring them into suspension, after which the organ was removed and washing, sedimenting, and decanting were continued until the supernatant fluid was clear. Then the sediment was placed in Petri dishes and examined under a low power of the microscope. In examining an entire organ such as the liver, it was ground up in a mortar, then suspended in water, and poured through a tea strainer to remove the larger particles. The filtrate was then washed, sedimented, and examined according to the method already described.

The chickens were killed by chloroforming and the examinations made as rapidly as possible. They were killed after periods varying from $2\frac{1}{2}$ hours to 57 days.

In an autopsy made on a chicken $2\frac{1}{2}$ hours after receiving ova the eggs were still in the crop and none had hatched. After 24 hours apparently all had hatched. Larvæ were found only in limited num-

TABLE I

No. of chicken.	Period after feeding ova.	Esophagus.	Crop.	Proventriculus.	Gizzard.	Small intestine.	Large intestine.	Ceca.	Cecal wall.	Lungs.	Liver.	Spleen.	Body cavity.	Remarks.
	hrs.													
320	$2\frac{1}{2}$ *days*	—	Unhatched ova.	—		—*								Chicken was given no food after receiving eggs.
321	1	—	—	—	—*	—*	Many.	Many.			—†	—†	—*	Organs normal.
275	1	—		—		Several.		+	—‡	—	—‡			Feces passed on autopsy table washed and sedimented. No larvæ or ova found.
322	2			—		Two.*	—	Many.*	—§					All organs normal.
276	2					—	—	+	—‡		—‡			" " "
277	3							+	—‡		—‡			Organs normal except one lung congested.
323	4	—	—	—		—*	—*	+	—§	—	—†		—*	All organs normal.
324	7	—	—	—		—*	—*	Many.	—§		—†		—*	Walls of ceca possibly slightly thickened and contained white specks. Mucosa congested. Other organs normal.
278	7							+	—‡		—‡			All organs normal except one lung congested.
325	9	—	—	—		—*	—	+	—§	+	—†			Mucosa of ceca congested. Scraping from congested place in lung contained anterior half of larva. Other organs normal.
279	10							+	+‡		—‡			Blackhead. All organs except ceca were normal.
280	11							Many.	+‡		—‡			Blackhead. All organs normal except ceca.

326	11	−	−	−		−*	−	+		−	−†		All organs normal except ceca.
281	14							+	+‡		−‡		Blackhead. All organs normal except one cecum. Mucosa covered with hemorrhages.
282	14							+	−‡				Blackhead. All organs normal except ceca.
327	16	−	−	−		−*	−	Many.	−§				Ceca showed certain abnormalities but other organs were normal.
285	18							+	−‡		−‡		All organs normal.
328	21	−	−			−	−	−*	−§				" " "
286	23							+	−‡		−‡		" " "
287	28							+	−‡		−‡		Mucosa of one cecum not normal.
288	29							+*	−‡		−‡		Mucous folds of ceca absent in places. Other organs normal.
332	35							7*					All organs normal.
335	49							−					" " "
336	51							1*					" " "
338	57							8*	−§				" " "

Three controls were killed, two on the 17th day and one on the 30th day. The organs were normal. The contents of the ceca were washed and sedimented but no larvæ were found.

* Contents washed, sedimented, and examined.

† Organs ground up, washed, strained, and sedimented.

‡ Fixed and stained sections.

§ Piece of fresh wall examined microscopically under pressure between two slides.

bers in the posterior part of the small intestine but in large numbers in the large intestine and ceca. After 2 days in one instance two larvæ were found in the small intestine but none elsewhere except in the ceca. The fact that larvæ in one instance were found in great numbers in the large intestine a day after feeding suggests that this is a site where they collect to migrate to the ceca. The point in the alimentary canal where the ova hatch has not been determined. It apparently does not take place in the crop. It seems probable that the small intestine is the site where the embryos emerge. No larvæ were found in lungs, liver, spleen, cecal wall, or body cavity. The larvæ in general had the same character as embryos within the egg.

In summarizing the observations on chickens killed the 3rd day and thereafter, it should be stated that worms were found only in the ceca and in three instances in the walls of the same. In one chicken (No. 325) a fragment of a larva was found in a scraping from a congested lung, but as no others were observed in further mounts, it seems most probable that this had been introduced by accident, possibly on instruments. Examinations in various instances of regions of the alimentary tract other than the ceca, of the lungs, the liver after being ground up, washed, strained, and sedimented, and of washings from the body cavity were uniformly negative for larvæ. Microscopic examination of the washed cecal wall were likewise negative. In two out of the twenty chickens examined after the 3rd day it was not possible to find worms in the ceca. Three controls examined were negative.

A surprising result of the feeding of ova was the light infestation resulting. It is believed that each bird received at least several hundred eggs, yet the number of worms found in certain instances was small. While the worms are still microscopic or visible to the naked eye with difficulty, it is not easy to form an estimate of the numbers present. However, in the case of Nos. 321, 322, 323, 324, 280, and 327, all killed on or before the 16th day, many larvæ were present. The presence of only three larvæ in a cecum of No. 286; two larvæ in the case of No. 287; and seven and eight worms in both ceca of Nos. 332 and 338 respectively, is evidence of the small infestation resulting. There are in all probability a number of factors involved in this result. Among these may be mentioned the artificial method of collecting

and incubating the ova. It is conceivable that this might have a deleterious influence on the vitality of the larvæ. Also the abnormal condition of the ceca and their contents in cases that develop blackhead might have an unfavorable influence on the larvæ.

It will be noted that worms reached maturity in 57 days. Taking 7 days, which was observed in experiments at room temperature as the shortest time for embryos to become fully developed, as the minimum period for development outside the body, the entire cycle would require 64 days.

Development of Larvæ (Figs. 1 to 11).

Embryos may be freed from ova by pressing on the cover-glass of a fresh mount and crushing the shell (Figs, 1 and 4). If uninjured on emerging they show an active wriggling motion. The maximum width occurs towards the anterior end and the body tapers in both directions, anteriorly to a rather blunt, rounded point, and posteriorly to a long, fine point. The length ranges from 290 to 340 microns, and the maximum width from 14 to 15.6 microns. The anus is situated 45 microns from the posterior end. The anterior end is nude and the mouth is subterminal, being located a little dorsal to the tip of the body. Lateral wings are present, and viewed from the side of the worm they appear as lines, most prominent in the middle region of the body and gradually shading out toward the extremities. The intestine is marked by a band of highly refractive granules extending from a point anterior to the middle of the body to near the anus. The esophagus is slightly bulbous, the bulb being elongated. The cuticula at the anterior tip of the body is slightly thickened.

Larvæ 1 and 2 days old show little change from the embryo just emerged (Figs. 2 and 3). The mouth is still subterminal. The rectum was wedge-shaped and in some instances it alternately expanded and contracted like a pulsating vacuole. A 2 day larva measured 320 microns long and 17 microns broad, and one at 3 days was 338 microns long.

Larvæ at the end of 7 days showed a marked growth (Fig. 7 and same stage as shown in Fig. 8). One measured 0.5 mm. in length and 28 microns broad. Those studied on the 9th and 10th days were somewhat more advanced (Figs. 8 and 9.) One measured 3 mm. In

some instances a pair of small round papillæ were observed for the first time on the anterior end opposite each other.

Larvæ collected on the 14th day were round and smooth on the anterior end with the exception of the presence of the papillæ already mentioned. Five ranged in size from 2.9 to 3.8 mm. A number of rather young larval stages were observed, a circumstance noted also on other occasions, indicating a considerable variation in the rate of growth.

By the 16th day lips were seen forming within at the anterior end, and chitinous structures similar to those of the adult were present in in the esophagus or in process of formation The papillæ already noted on the anterior end were present. These are always seen in optical section of the worm on either side some distance from the mouth, but there may be others not visible on the upper and lower surfaces. At about this time when the lips become free a molt takes place, but specimens showing this have not been encountered. Larvæ measured ranged from 3.2 to 3.9 mm. long.

On the 18th day specimens were found showing further development. Sexes were differentiated, the males being provided with a bursa and papillæ. Larvæ ranged in size from 2.6 to 4 mm. long. On the 29th day worms had reached a maximum of 4.5 mm. in length. By the 35th day some worms were about half grown, and by the 57th day they had apparently reached full development.

SUMMARY.

In observations on the development of the ova of *Heterakis papillosa* in cultures, it was found that they failed to develop at a temperature ranging from 2.5–8°C., but developed slowly at a temperature of 11.5–13.5°C. The minimum temperature for development seems to lie between 8° and 11.5–13.5°C. At temperatures ranging in various cultures from 18–29°C. ova developed to their final stage in 7 to 12 days.

Undeveloped ova subjected to a freezing temperature for a period of 4 days were viable at the end of that time. Fully developed ones remained alive when exposed out of doors for a period of 7 days at a temperature ranging from 5–62°F.

Undeveloped ova survived desiccation at room temperature for a period of 16 days, but not for 41 days. Fully developed eggs were alive after desiccation for 18 days, but not after 49 days. In another instance they were no longer viable after 10 days.

Embryos within ova kept in physiological salt solution at room temperature survived during a period of a little over 12 months. Fully developed ova kept in soil outdoors under circumstances approaching natural conditions contained living embryos after a period of 8 months.

From a study of a series of artificially infested chickens killed at short intervals it appears that the ova of *Heterakis* hatch in the small intestine and the larvæ pass by way of the small and large intestines to the ceca where they undergo development to maturity. Larvæ found in the mucosa of the ceca were not in an encysted condition.

Feeding of numerous artificially incubated ova may lead to a light infestation, the cause of which has not been definitely determined.

A period of 57 days was required for larvæ to reach maturity in a host. The entire cycle from egg to adult requires a minimum time of about 64 days.

A brief study of the growth and development of larvæ within the host has been made. No evidence was found of a migration through the tissues. A few penetrate into the mucosa of the ceca.

EXPLANATION OF PLATE 18.

Drawings made with camera lucida from fresh material.

FIG. 1. Embryo from an egg in a culture. Magnification about 196.

FIG. 2. Larva, 1 day old. Rendered immobile by adding alcohol and glycerol to mount. Magnification about 196.

FIG. 3. Larva, 2 days old, showing lateral wing. Magnification about 196.

FIG. 4. Anterior end of specimen shown in Fig. 1. Shows esophagus and subterminal position of mouth. Magnification about 834.

FIG. 5. Anterior end of a larva 3 days old. Magnification about 834.

FIG. 6. Anterior portion of body of a larva 4 days old, showing bulbous esophagus and anterior end of intestine. Magnification about 387.

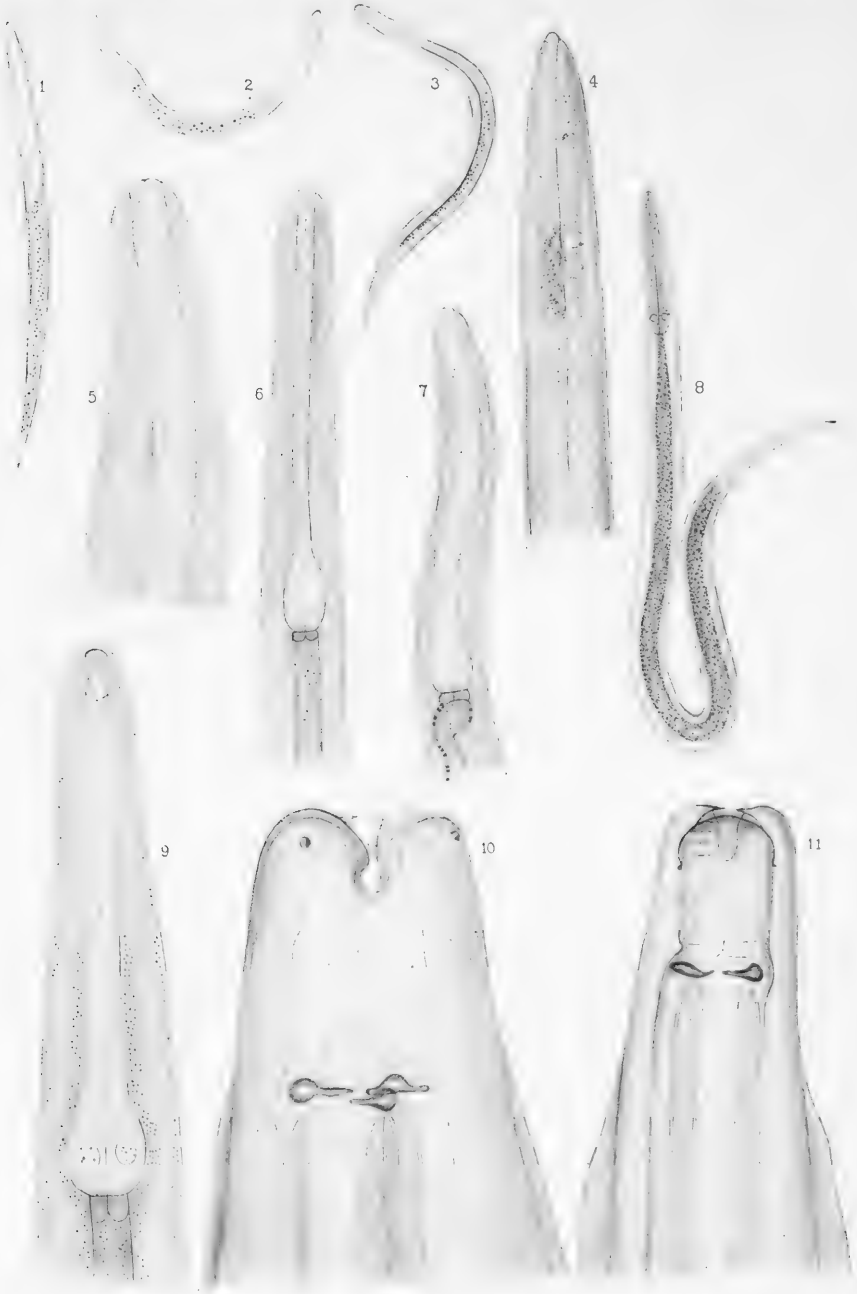
FIG. 7. Anterior region of body of a larva 7 days old. Shows bulbous esophagus. Magnification about 425.

FIG. 8. Larva 10 days old. Shows bulbous esophagus and intestine crowded with granules. Magnification about 46.

FIG. 9. Anterior end of same larva. Shows a differentiation of pharynx. Magnification about 182.

FIG. 10. Anterior end of worm 29 days old. Shows the two ventral lips with teeth and papillæ, the pharynx and chitinous structures of same, and of esophagus. Magnification about 660.

FIG. 11. Anterior end of a worm 35 days old. Dorsal view showing three lips. Magnification about 387.



(Graybill: *Heterakis papillosa* in the fowl.)

LYMPHOPENIA FOLLOWING EXPOSURES OF RATS TO "SOFT" X-RAYS AND THE β-RAYS OF RADIUM.

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Murphy and his collaborators have referred to a difference between the effects of large and small doses of x-rays on the circulating lymphocytes of the rat, large doses producing a diminution, and small doses an increase in their numbers (1, 2). The dose required for the latter they refer to as a "stimulating" dose. Our own observations (3) upon the effects of moderately penetrating x-rays, ranging in quantity from very small to quite large doses, showed that, in all cases, there was a preliminary disappearance of lymphocytes from the ciculation, generally observable an hour after the exposure to the x-rays. In view of the fact that in many of Murphy's experiments x-rays of a very easily absorbed type were used, it occurred to us that it might be useful to extend our observations with "soft" x-rays similar in character to those used by Murphy, and to supplement them by the use of β-rays of even less penetrating power than these "soft" x-rays. From the data below, it will be seen that, as in our previous experiments, an initial fall in the number of circulating lymphocytes occurs, provided that the blood observations are done soon after the exposure to the radiation.

In view of these findings, it appears that the terms "destroying" and "stimulating" doses as used by Murphy may prove misleading, for they are apt to give the impression that essentially different effects are observed after short and prolonged exposures to x-rays. This, however, has not been our experience, for whereas the lymphopenia following a large dose (4) may continue for as long as a week or 10 days, a small dose produces a lymphopenia which lasts but a few hours.

It is in our opinion important to bear in mind that the lymphocytosis which occurs after a small dose or after a large dose of these radiations follows a primary lymphopenia.

Experimental Conditions.

X-Rays.—The "soft" x-rays used for our present purpose were those emitted by a Coolidge tube at an alternative spark-gap of 0.75 cm. between spheres 5 cm. in diameter; this is equivalent to $\frac{3}{4}$ inch between a point-to-point spark-gap. The dose of radiation admin-

TABLE I.

Rats Exposed to "Soft" X-Rays for 12″.

Weight of rat.	Lymphocytes per c. mm. before exposure.	Lymphocytes per c. mm. after exposure.	Fall.
gm.			*per cent*
118	28,700	10,400	63.6
108	18,400	9,800	46.8
130	27,000	8,800	67.5
About 100.	32,400	12,500	61.4
	30,400	14,300	53.4
	18,000	8,500	55.6
Average			58

TABLE II.

Rats Exposed to β-Rays for 34 Minutes.

Weight of rat.	Lymphocytes per c. mm. before exposure.	Lymphocytes per c. mm. after exposure.	Fall.
gm.			*per cent*
60	5,100	2,700	47
90	8,600	5,900	29
160	8,000	5,200	35
105	19,300	10,400	46
95	13,400	9,100	32
90	7,200	4,500	37
Average			38

istered to the rats was equivalent to what is referred to in our paper as a twelve″ dose; this corresponds approximately to $\frac{1}{150}$ of a rad. Our previous experimental finding was that when a rat was exposed to x-rays of a moderately penetrating character for 12″, an average reduction of 50 per cent in the number of circulating lymphocytes occurred 1 hour after the irradiation. It will be seen from the data

in Table I that, with an equivalent exposure to "very soft" x-rays, the average reduction in the first six rats selected for the test was 58 per cent.

β-Rays.—Two varnished radium applicators 4 by 4 cm. were used, each containing 80 mg. of radium bromide (Ra $Br2H_2O$); the animals were confined in a box 15 by 15 by 15 cm.; the radium was let in to the roof of the box, so that it was 16 cm. from the floor, nothing intervening between the radium and the animal on the floor of the box. The exposure lasted 34 minutes, this being the time necessary for the surface dose to be $\frac{1}{150}$ of a rad, the same as in the x-ray experiment.

Blood examinations were made immediately before the exposures to radium, and again 1 hour after the end of the exposures. Six rats were used; the results will be seen in Table II.

The smaller percentage decrease observed with β-rays is probably to be attributed to their smaller penetrating power than the "soft" x-rays. It seems to us that the experimental results indicate that, had Murphy done blood counts soon after his x-ray exposures, he would have observed this lymphopenia in his small dose studies.

BIBLIOGRAPHY.

1. Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800.
2. Thomas, M. M., Taylor, H. D., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 75.
3. Russ, S., Chambers, H., Scott, G. M., and Mottram, J. C., *Lancet*, 1919, i, 692.
4. Mottram, J. C., and Russ, S., *Proc. Roy. Soc. London, Series B*, 1917–18, xc, 1.

THYROIDECTOMY AND PARATHYROIDECTOMY WITH RELATION TO THE DEVELOPMENT OF IMMUNE SUBSTANCES.

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Plates 19 and 20.

(Received for publication, April 12, 1921.)

An investigation of the literature discloses immediately the unsettled state of the problem of thyroidectomy and parathyroidectomy with relation to the development of immune substances. The reports have been concerned particularly with the site of antibody production. Removal of important organs, preceded and followed by an investigation of natural or acquired antibodies of various kinds, has been resorted to by numerous investigators in the hope of finding one organ in the body which has an undoubted relation to the development of immune bodies, or with a view to prove that no particular one is responsible, and that the phenomenon is entirely a humoral one. Gates performed partial adrenalectomy and studied the development of anti-hen hemolysin, with negative results. Hektoen excised spleen, pancreas, and portions of the liver and intestinal tract. Splenectomy caused diminution in the development of immune substances, while the other procedures had no definite effect. Although thyroidectomy has been performed by a number of investigators on various animals, *viz.*, dog, fox, chicken, horse, and rabbit, yet the relation of the thyroid gland to the development of immune bodies remains an unsettled question because of conflicting results. The relation of the parathyroids in particular to the production of immune substances is still more obscure.

HISTORICAL.

Hektoen and Carlson worked with dogs, but because of the practical impossibility of removing thyroid without parathyroids, found conditions unfavorable for the study at that time. In 1907, Fassin, working with thyroidectomized

dogs, and rabbits, found a marked diminution of the natural hemolytic as well as the bacterial alexin, but never complete disappearance. Administration of thyroid substance increased the concentration of complement in the serum. This author found that death of the animals followed thyroidectomy rather rapidly, a result not in accord with those of other observers, including ourselves, which leads us to believe that the parathyroids also were removed. Marbé, in 1909, thyroidectomized four dogs and bled them when they developed symptoms. He found a definite diminution of the opsonic power of their serum against staphylococci and tubercle bacilli. He admits that he removed parathyroids as well. He also found an increase in the opsonic power of the serum of a myxedematous patient. In 1910, Fjeldstad, working with rabbits, came to the conclusion that "removal of the thyroids from the rabbit does not, at least during the first month, appreciably affect the formation of immune bodies (more specifically the agglutinins)." Dogs were thyroidectomized by Frouin, and then fed with calcium and magnesium salts. He studied the production of anti-horse and anti-rabbit hemolysin. As a matter of fact, the parathyroids were removed as well. Those animals that developed tetany, some even as long as 14 months after operation, although treated for 3 months with calcium and magnesium salts, showed a diminished production of hemolysin against horse and rabbit blood. Here again it is apparent that parathyroids as well as thyroid were removed.

Launoy and Lévy-Bruhl (1913) worked on five chickens, several of which they thyroidectomized, and in the remainder removed the entire thyroid and part of the parathyroids. They concluded that the thyroid gland plays no rôle either in the resistance of these animals to infection with *Spirochæta gallinarum*, or in the rapidity and intensity of antibody production against this organism. They quote Lerda and Diez to the effect that thyroidectomized guinea pigs were found to be equally, or more resistant to intoxication by tetanus and diphtheria toxins, strychnine, and caffeine than normal animals. However, Houssay and Hug more recently found thyroidectomized horses markedly sensitive to diphtheria toxin. Again, in 1915, Launoy and Lévy-Bruhl conclude that thyroidectomy in chickens does not inhibit the resistance or the antibody production against *Spirochæta gallinarum*. They worked with the protective power of the acquired immune serum against *Spirochæta gallinarum*. In 1915, Hektoen and Curtis thyroidectomized dogs immediately after injecting them with rat blood. Death followed in 10 days—from tetany—a fact which leads one to believe that the parathyroids also were removed. Following the operation no "variation in the usual course and amount of antibody production" was noted p to the time of death.

One of the most recent investigators of the subject is Garibaldi. In January, 1920, he published his results on a study similar to, but not so extensive as ours. He performed thyroidectomy in rabbits, and studied the development of anti-sheep hemolysin. He used seven animals, three of which were controls. It is

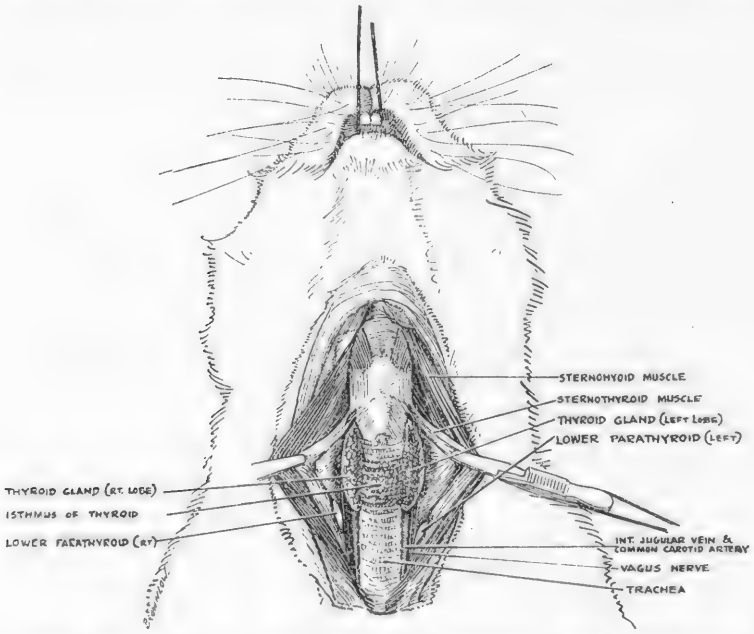
not clear from his paper whether he removed parathyroids as well, nor does he state the concentration of the cell suspension used. The injections were made intraperitoneally and repeated twice at intervals of 7 days. In his titration he used 1 cc. of a 5 per cent suspension of sheep blood corpuscles and a "constant and sufficient quantity of complement." The first and only titration was made 12 days after the last injection—exactly 1 month after the operation. The average titer of the series was 1:7,000 in the thyroidectomized as compared with 1:1,200 in the controls. He comes to the conclusion that the heterohemolytic titer of the serum of the thyroidectomized rabbits immunized during the month following the operation is definitely higher than that of the control animals, and that thyroidectomy seems to favor the formation of antibodies. He leaned for confirmation of his conclusions upon the results of Frouin, and also to a certain extent upon those of Launoy and Lévy-Bruhl. But the latter replied vigorously to this (1920), repeating that their results did not warrant the statement that thyroidectomy favored hyperresistance to infection. Garibaldi's conclusions were based on an experiment with seven animals. The striking feature in his work is the very slight variation and the uniformly high hemolytic titer which he obtained. It is well known that rabbits, normal or otherwise, respond in an extraordinarily variable manner to the injection of sheep blood. A glance at our charts, representing many titrations of the serum of numerous animals will demonstrate the tremendous variations. Some rabbits are very poor producers of hemolysin, the titer of their serum remaining persistently very low, despite frequently repeated injections of blood. This has been the experience of Koopman and others.

Houssay and Hug (1920) recently thyroidectomized young horses. One of the animals immunized to *B. typhosus* gave an agglutinating serum with a titer of 1:50,000 which was considered exceptionally high.

In going over the work of the above quoted investigators we were uncertain in many cases whether they removed thyroid only, or parathyroids as well. The anatomy of the thyroparathyroid apparatus varies in different animals. Since there is good reason to believe that these glands differ in their development and function (Hagenbach), it is reasonable to assume that their removal would have a variable effect, if any, upon the body and its functions in different species. It is important therefore, to know exactly what has been removed. It seems to us that lack of clearness on this point accounts, in part at least, for the variable and often contradictory results obtained. Therefore, a few words on the anatomy of the thyroparathyroid apparatus in the rabbit are not out of place.

Anatomy.

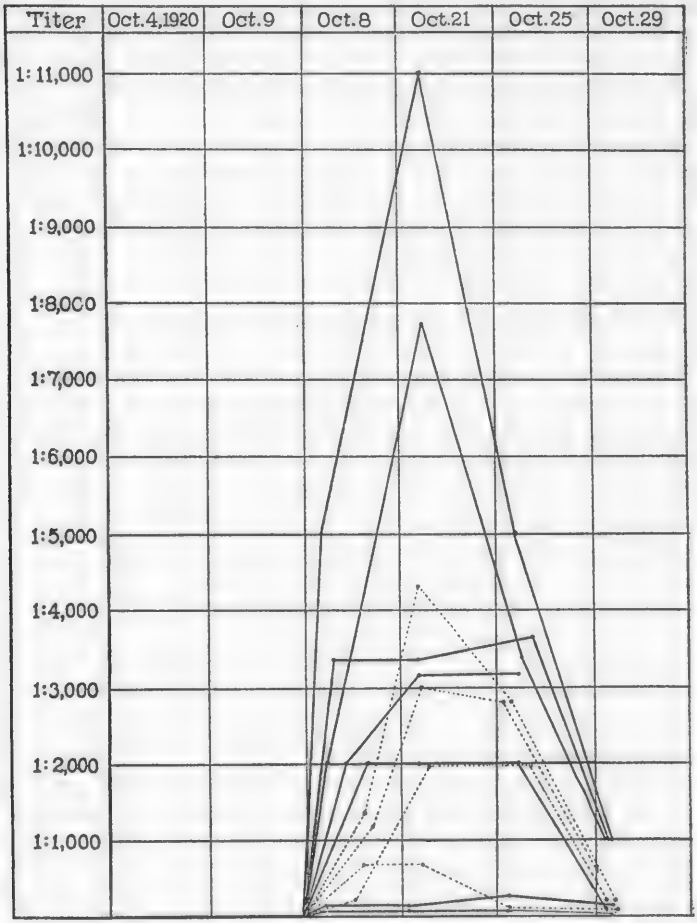
In the rabbit, as in most other animals, the thyroid gland is composed of two lobes and an isthmus (Text-fig. 1). The lateral lobes lie on either side of the larynx and upper portion of the trachea. The tip of the upper pole is hidden beneath the attachment of the sternothyroid muscle to the thyroid cartilage. The isthmus is thin and covers



TEXT-FIG. 1. In this rabbit the upper parathyroid glands were situated within the parenchyma of the lateral lobes and do not show in the drawing.

usually two or three rings of the trachea immediately below the level of the cricoid cartilage. There are, as a general rule, four parathyroid bodies—two upper and two lower. The position of the upper two varies greatly. In some cases they are situated outside the capsule of the thyroid, internal or posterior to the lateral lobes (Fig. 1), while in a great percentage of cases they are situated either within or under the capsule of the thyroid, or are actually embedded in some portion of

the thyroid parenchyma (Fig. 2). In the rabbit, therefore, it is practically impossible, except in an occasional case, to remove the thyroid gland without also removing the upper parathyroid bodies. The



TEXT-FIG. 2. Thyroidectomy with partial parathyroidectomy. Graphic representation of all the titrations in Series 1. ——— Thyroidectomized rabbits. ..... Normal controls.

lower parathyroids are usually easily recognizable as small fusiform bodies approximately 8 by 2 mm. in diameter. Ordinarily they are light pink in color, but sometimes white or red. They are situated under cover of the sternothyroid muscle in intimate relation with the great vessels of the neck. Their position varies from immediately below the tip of the lower pole of the lateral lobe of the thyroid to several centimeters below it—the left one as a rule being situated about 1 cm. below the level of the right (Text-fig. 1).

On account of the anatomical relations of the thyroid and parathyroids, we divided our experiments into three parts: (*a*) thyroidectomy with partial parathyroidectomy; (*b*) partial parathyroidectomy alone (removing only the lower glands); and (*c*) complete thyroparathyroidectomy.[1]

Thyroidectomy with Partial Parathyroidectomy.

The rabbits used were approximately 6 months of age and averaged 2.2 kilos in weight. In the first series twelve rabbits were used. The natural anti-sheep hemolysin was first determined, and in no case was found to give complete hemolysis in a dilution even of 1:5, with 0.5 cc. of inactivated serum, 0.5 cc. of 5 per cent cell suspension, and two units of complement. Expecting fatalities, we thyroidectomized seven animals and used the remaining five as controls. Of the latter, three were operated upon for exposure of the gland, without removal. All of the thyroidectomized animals lived, but one control died. Other than slight depression during the first few days after the operation, and slight irritability in one or two animals, nothing abnormal was noted in those that were thyroidectomized. The animals were bled and the serum was titrated 24 hours after the operation. No definite change in the natural hemolysin was noted. 5 days after the operation the rabbits had recovered completely. At that time they were given the first intravenous injection of 2 cc. of a 50 per cent suspension of washed sheep blood corpuscles. This was followed 2 days later by an injection of 3 cc. of a similar suspension, and 2 days after that the animals received 5 cc., the final injection. 3 days after the last injec-

[1] All operations were performed under ether anesthesia.

tion, and 12 days after the operation, the animals were bled and their serum was titrated for its hemolytic activity. In all our titrations we used 0.5 cc. of a 5 per cent suspension of washed sheep blood corpuscles, two units of complement, and 0.5 cc. of inactivated rabbit serum in increasing dilutions, and incubated them in a water bath at 37°C. for 1 hour. The highest dilution of the serum which then showed complete hemolysis was recorded as the titer. Titrations were repeated in most instances every 2 days until the height of antibody production had been reached and definitely passed. The graphic record of the titrations shows the great variation of the titers of the sera both of

TABLE I.

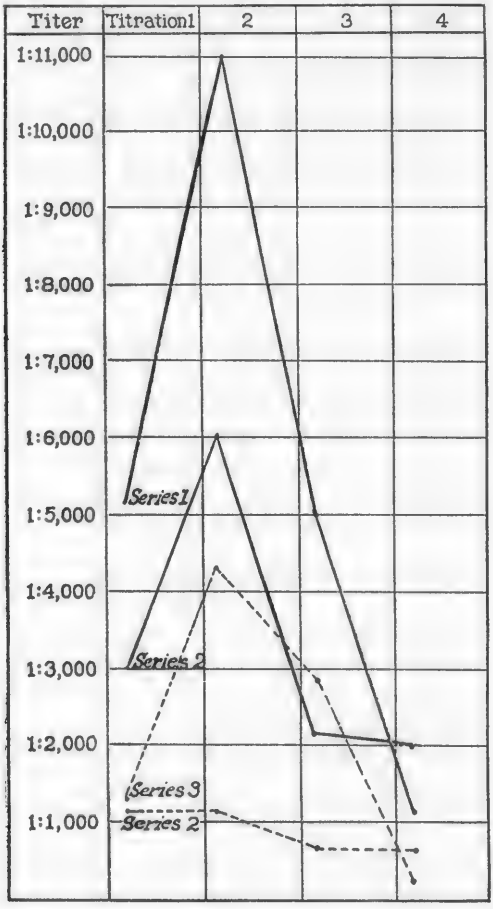
Thyroidectomy with Partial Parathyroidectomy.

Titration No.	Maximum titer.		Average titer.	
	Thyroidectomized rabbits.	Controls.	Thyroidectomized rabbits.	Controls.
		Series 1.		
1	1:5,200	1:1,400	1:2,500	1:775
2	1:11,000	1:4,400	1:3,850	1:2,450
3	1:5,000	1:2,800	1:2,600	1:1,900
4	1:1,200	1:400	1:575	1:250
		Series 2.		
1	1:3,000	1:1,200	1:1,400	1:700
2	1:6,000	1:1,200	1:2,350	1:875
3	1:2,400	1:600	1:1,250	1:350
4	1:2,000	1:600	1:850	1:250
5	1:1,800	1:600	1:700	1:250

the thyroidectomized rabbits and of the controls. The lowest titer of the thyroidectomized rabbits was as low as that of the controls, but in every titration the maximum titer in the thyroidectomized group was considerably higher than the maximum of the controls (Table I and Text-figs. 2 and 3). In the various titrations the average titer of the serum of the thyroidectomized animals was definitely higher than that of the controls (Text-fig. 4).

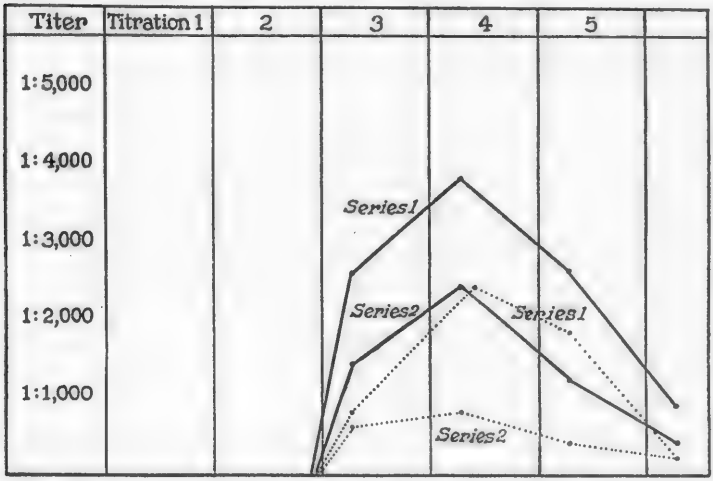
In the second series in which thyroidectomy with partial parathyroidectomy was performed fifteen animals were used. Three died, leaving seven thyroidectomized rabbits and five controls, in three of

which the thyroid was exposed but not removed. The injections and titrations were performed in the same manner and at the same intervals as in Series 1. The results of the titrations are recorded graphi-



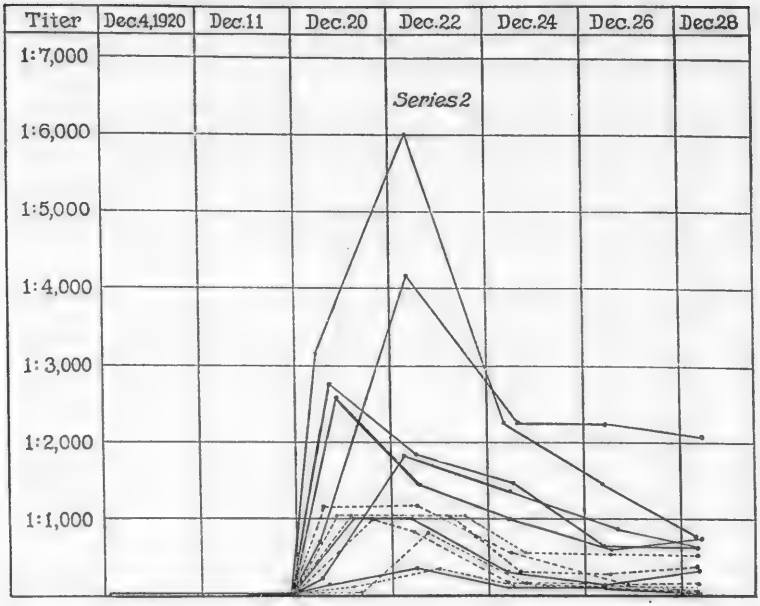
TEXT-FIG. 3. Thyroidectomy with partial parathyroidectomy. Graphic comparison of maximum titers in the various titrations of Series 1 and 2. ——— Thyroidectomized rabbits. ..... Normal controls.

cally in Text-fig 5. Here, again, the lowest titer of the thyroidectomized group was as low as that of the controls, but in every titration the highest titer of the serum of the thyroidectomized animals was higher than that of the controls. A comparison of the average titers of the sera shows that of the thyroidectomized animals was about three times as great as that of the controls (Table I, Series 2). In this series also, the variation in the titer of the different sera was very great, as can be seen in the graphic representation of the titrations (Text-fig. 5).



TEXT-FIG. 4. Thyroidectomy with partial parathyroidectomy. Graphic comparison of average titers in all the titrations of Series 1 and 2. ——— Thyroidectomized rabbits. ..... Normal controls.

In a third series we repeated Garibaldi's experiment, giving the injections of sheep blood intraperitoneally as he did. We used ten rabbits, five of which were thyroidectomized and partially parathyroidectomized. Not knowing what suspension of sheep blood he used, we washed the corpuscles and made them up to blood volume. In all other respects we repeated his work to a detail. However, the titer of the hemolysin produced was very much lower in our series than in his, and the thyroidectomized rabbits and controls produced



TEXT-FIG. 5. Thyroidectomy with partial parathyroidectomy. Graphic representation of all the titrations in Series 2. ——— Thyroidectomized rabbits. ..... Normal controls.

TABLE II.

Thyroidectomy with Partial Parathyroidectomy Followed by Three Intraperitoneal Injections of Sheep Blood at Intervals of 1 Week (Garibaldi's Method).

Date of titration.*	Maximum titer.		Average titer.	
	Thyroidectomized rabbits.	Controls.	Thyroidectomized rabbits.	Controls.
1921				
Mar. 12	1:600	1:800	1:350	1:500
" 14	1:1,000	1:1,000	1:450	1:450
" 16	1:800	1:1,000	1:475	1:600
" 18	1:400	1:500	1:300	1:350

* Last injection Mar. 1. First titration 12 days later.

approximately an equal amount of hemolysin. Only in two of the controls and in one of the thyroidectomized animals did the titer reach 1:1,000 at the height of production (Table II). This part of our work did not confirm Garibaldi's findings.

Complete Thyroparathyroidectomy.

By the above term we mean removal of all grossly recognizable thyroid and parathyroid tissue confirmed at necropsy by a careful investigation of the neck and chest for accessory parathyroid tissue. Naturally enough, serial sections of the neck and chest were not made to rule out possible inclusion of parathyroid gland in other tissues or organs. In a few cases small suspicious bodies were discovered at necropsy low in the front of the neck, but invariably they turned out to be lymph nodes. Four sets of rabbits were employed for this part of the experiment—a total of 60 rabbits being used. Like Carlson and Woelfel who thyroparathyroidectomized foxes, we found complete recovery to occur after extreme symptoms of tetany with violent and prolonged convulsions, although the animals were expected to die. The symptoms developed in less time in our rabbits than in foxes. There was also a tendency to periodicity of the symptoms, and in some the symptoms were delayed, while in an occasional animal they did not develop at all. Other investigators working with various animals report similar experiences. Farner and Klinger, studying the relation between parathyroids and tetany in the rat, found that some recovered even after apparently complete removal. Ferreira de Mira found that guinea pigs may survive complete thyroparathyroidectomy, some living as long as 57 days after the operation. So it would seem that a certain proportion of animals may live after complete thyroparathyroidectomy just as they do after complete adrenalectomy. Whether the explanation is that there are undiscoverable accessory glands, or that other organs take over the function, we are not prepared to state.

Twelve rabbits were used in the first series. Six were completely thyroparathyroidectomized, and of these four survived. The two died of tetany, while the others developed varying degrees of it. Since we did not know how long the animals would live, they were given one intravenous injection of 7 cc. of a 50 per cent suspension of

sheep blood 20 hours prior to the operation. The results of the titrations, which were made at intervals of 2 days, can be seen in Table III, Series 1. All the titers were low in this case, because only one injection was given. A striking difference is seen, nevertheless, between the thyroparathyroidectomized animals and the controls. In every titration the maximum titer reached by the thyroparathyroidec-

TABLE III.

Complete Thyroparathyroidectomy.

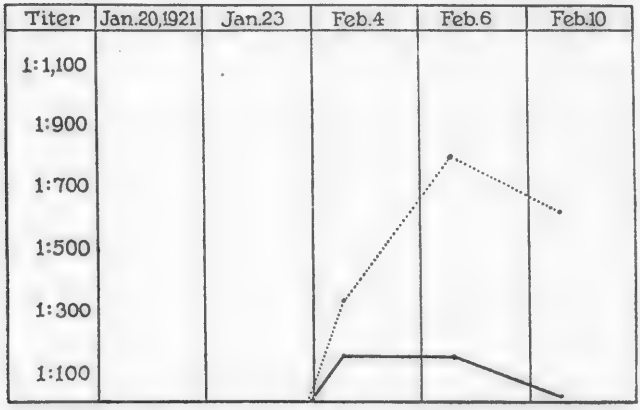
Titration No.	Maximum titer.		Average titer.	
	Thyroparathyroidectomized rabbits.	Controls.	Thyroparathyroidectomized rabbits.	Controls.
		Series 1.		
1	1:200	1:400	1:150	1:290
2	1:200	1:1,000	1:150	1:800
3	1:50	1:800	1:15	1:500
		Series 2.		
1	1:200	1:2,000	1:150	1:1,500
2	1:400	1:5,000	1:300	1:2,050
3	1:200	1:1,000	1:100	1:600
		Series 3.		
1	1:800	1:1,200	1:400	1:725
2	1:2,000	1:6,000	1:1,800	1:3,700
3	1:1,800	1:3,400	1:1,300	1:1,500
		Series 4.		
1	1:200	1:1,600	1:120	1:1,100
2	1:800	1:2,600	1:340	1:1,900
3	1:1,000	1:2,200	1:370	1:1,650
4	1:400	1:1,400	1:170	1:1,100

tomized animals was very low compared with that of the controls, and the average titer at the height of production was less than one-fifth that of the controls. A graphic comparison of the average titers in this series is given in Text-fig. 6.

Since some parathyroidectomized animals survived, injections were given in the remaining three series at the same time and in the same

amounts as in the thyroidectomy experiments; *viz.*, 2, 3, and 5 cc. at intervals of 2 days, beginning 5 days after the operation.

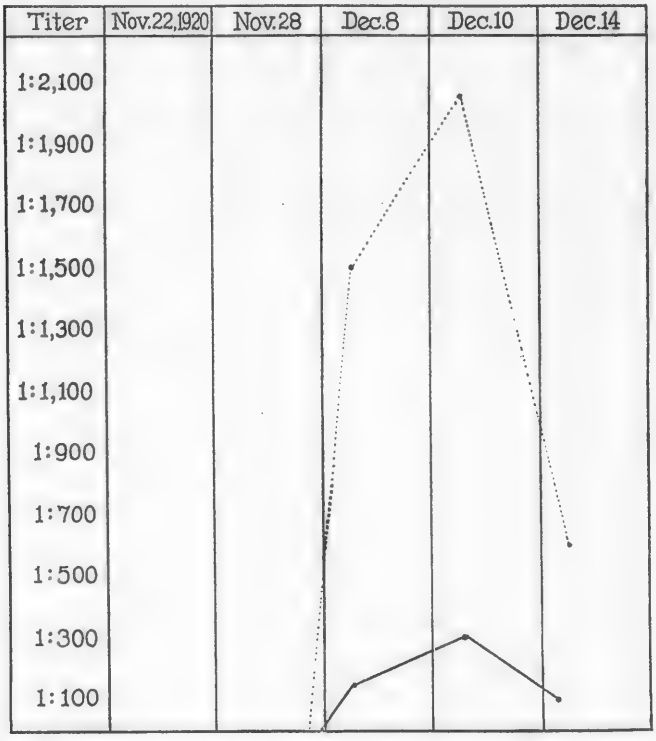
In Series 2, eleven rabbits were completely thyroparathyroidectomized. All developed severe tetany, and only two survived. The latter also had very severe symptoms, but recovered. The titrations in this series also show relatively low titers for the thyroparathyroidectomized animals. In Text-fig. 7 the comparison of the average titers in this series is represented graphically (see also Table III, Series 2). At the height of antibody production the average titer of the thyroparathyroidectomized rabbits was to the controls as 1 is to 7.



TEXT-FIG. 6. Complete thyroparathyroidectomy. Graphic comparison of average titers in Series 1. ———— Completely thyroparathyroidectomized rabbits. ..... Normal controls.

In Series 3, four rabbits were thyroparathyroidectomized and four normal animals were used as controls. In this series we gave several subcutaneous and intravenous injections of a 1.5 per cent solution of calcium chloride in the hope of saving more animals. The same dose was given to the controls also. The animals had severe attacks of tetany, but three survived, and after the third day had no more symptoms. For results of titrations see Table III, Series 3, and Text-fig. 8. Here again there was relatively less antibody production in the thyroparathyroidectomized group, though the difference was not

so striking as in the other two series. In this series the average titer of the thyroparathyroidectomized animals and of the controls was higher than in the others. Whether this is attributable to the administration of calcium we do not know. At the height of antibody pro-

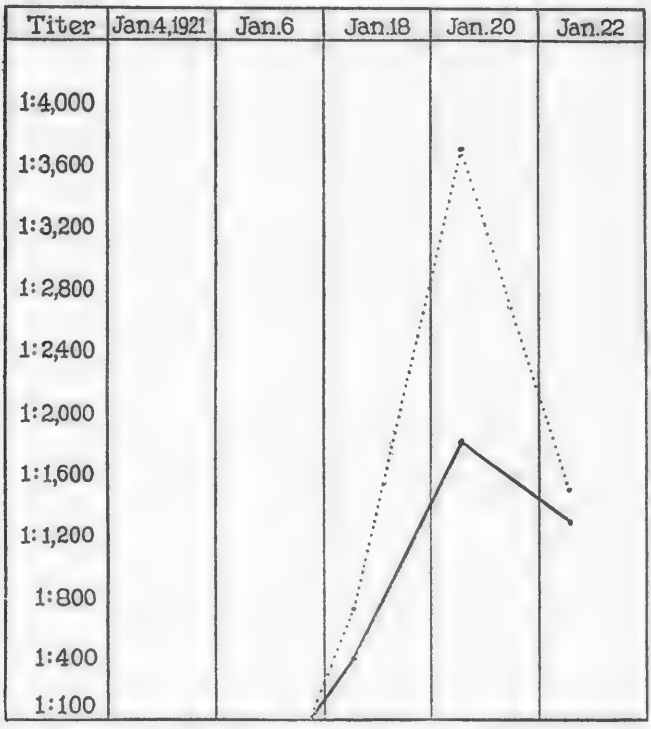


TEXT-FIG. 7. Complete thyroparathyroidectomy. Graphic comparison of average titers in Series 2. ———— Completely thyroparathyroidectomized rabbits. ..... Normal controls.

duction the average titer of the thyroparathyroidectomized rabbits was to that of the controls as 1 is to 2.

In Series 4, eight rabbits had the thyroid and parathyroids completely removed, five had only the lower parathyroids removed, and

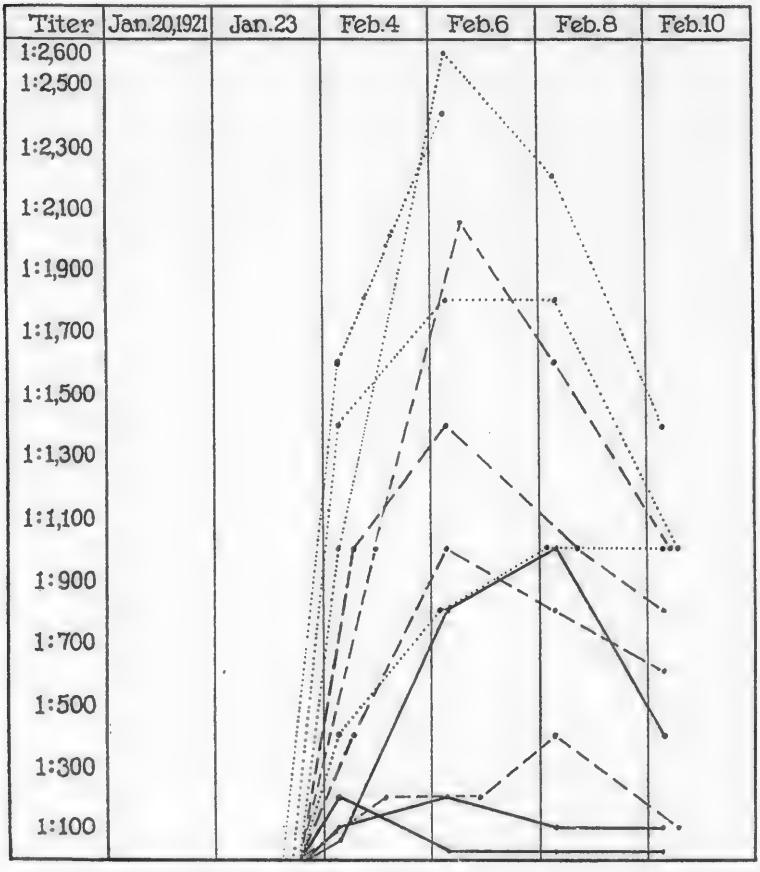
five normal animals were used as controls. In as short a time as 8 hours some of the aimals showed definite signs of tetany. Within 24 hours nearly all the animals completely thyroparathyroidectomized developed varying degrees of tetany, and some died. Some received



TEXT-FIG. 8. Complete thyroparathyroidectomy. Graphic comparison of average titers in Series 3. ———— Completely thyroparathyroidectomized rabbits. ..... Normal controls.

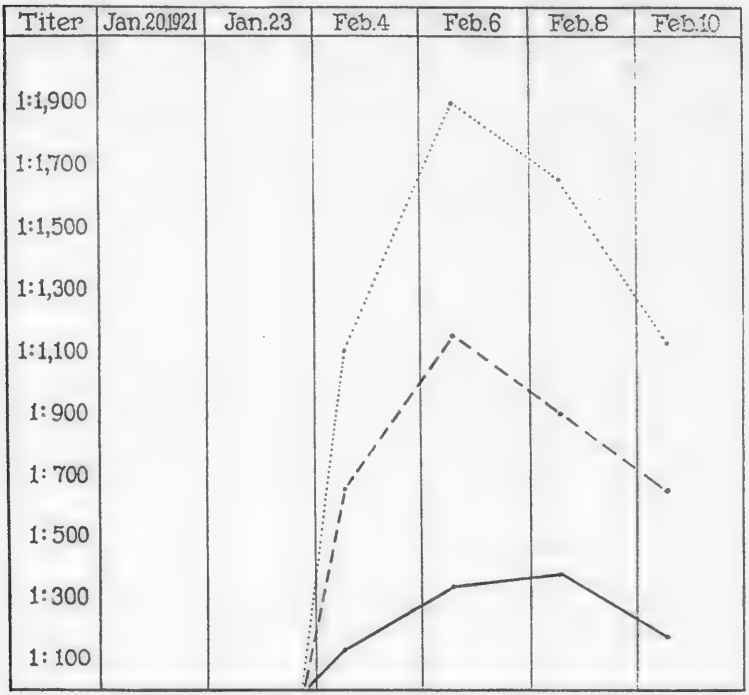
calcium chloride injections intravenously, but the tetany continued and they died, while other received no injections, had very severe tetany, yet survived. Of the eight thyroparathyroidectomized animals only three recovered. Five more were operated upon 2 days later, and of these only one survived. This one also had very severe

attacks of tetany but recovered and remained well. It was autopsied recently and not a vestige of thyroparathyroid tissue was found. For the results of the titrations, see Table III, Series 4, and Text-fig. 9. Those animals that were partially parathyroidectomized



TEXT-FIG. 9. Complete thyroparathyroidectomy. Graphic representation of all the titrations in Series 4. ———— Completely thyroparathyroidectomized rabbits. ---------- Partially parathyroidectomized rabbits. ..... Normal controls.

developed a little irritability and depression, but all survived. In this series the completely thyroparathyroidectomized animals developed the least amount of antibodies, while next in order came the partially parathyroidectomized, and finally the controls. Text-fig. 10



TEXT-FIG. 10. Complete thyroparathyroidectomy. Graphic comparison of average titers in Series 4. ———— Completely thyroparathyroidectomized rabbits. ---------- Partially parathyroidectomized rabbits. ..... Normal controls.

is a graphic comparison of the average titers in the various titrations of this series. The average titer of the completely thyroparathyroidectomized animals was approximately one-sixth that of the controls.

Finally, we took six thyroparathyroidectomized animals from Series 2 and 3, operated upon from 2 to 3 months previously. Three of them were fed with parathyroid tablets (Armour), 0.15 grain daily for a week and were then given a series of three injections, this time of bovine blood corpuscles, 2, 3, and 5 cc. of a 50 per cent suspension, at intervals of 2 days. No difference was found in the development of hemolysin between those upon which opotherapy was practised and the controls, but it is very interesting to note that all six were very low in their development of anti-sheep hemolysin, the maximum titer at the height of production being only 1:200, while in a series of normal animals injected in the same way the average titer was 1:1,000 We have no way of knowing, of course, whether we were really administering parathyroid gland and whether the dose was sufficient to have any effect. We did not give the gland by enteric capsules but gave it by mouth, which allowed gastric secretions to act on it before it was absorbed.

Titrations were made by the colorimetric method of the hydrogen ion concentration of the serum of rabbits prior to and after thyroidectomy and complete thyroparathyroidectomy. In all cases this was found to be within normal limits (7.6 to 7.8). Hastings and Murray working recently with the plasma of parathyroidectomized dogs obtained the same result.

DISCUSSION.

Hektoen, taking advantage of the work on iodobenzoic compounds by Loevenhart and his associates, tested the effect of the administration of iodoxybenzoate of soda on the production of antibodies. He found that dogs which had received iodoxybenzoate produced anti-goat hemolysin of a higher titer than that of dogs which had received injections of iodobenzoate, and higher than that of control dogs which had not received any injections. He concluded that the oxygen element was responsible for this difference. The thyroid gland is supposed to have a definite relation to the process of internal oxidation in the body. Yet in our experiments the removal of the entire thyroid gland with the upper parathyroid glands did not inhibit the production of hemolysin. In fact, the average titer of the serum of thyroidectomized animals intravenously injected with sheep blood was higher

than that of the controls. In view of the divergent results the question of the effect of internal oxidation on the development of immune bodies remains open.

Is the general disturbance created in these animals responsible for the effect on antibody production? During the first few days of the operation the completely thyroparathyroidectomized rabbits showed signs of much greater disturbance than did the thyroidectomized animals, but by the time the injections were begun the condition of both types of animals was approximately the same, and both gradually developed moderate cachexia. Yet, the thyroidectomized rabbits developed a hemolysin of relatively very high titer compared with the control animals, while the thyroparathyroidectomized animals developed a very low titer hemolysin compared with that of normal controls.

SUMMARY AND CONCLUSIONS.

1. After thyroidectomy with partial parathyroidectomy the maximum and average hemolytic titers of the sera of rabbits injected intravenously with sheep blood are equal to or higher than those of normal animals similarly injected.

2. Thyroidectomy with partial parathyroidectomy does not inhibit antibody production. This fact is in accord with the results of Garibaldi, Launoy and Lévy-Bruhl, Lerda and Diez, and others.

3. Thyroidectomy with partial parathyroidectomy does not cause serious disturbance in the adult rabbit. If the operation is performed properly, the animals survive and only moderate cachexia develops in time.

4. After complete thyroparathyroidectomy a small proportion of the animals survive even after developing very severe tetany. Those that recover do not show further signs of serious disturbance, but in time develop a moderate degree of cachexia no greater than that of the thyroidectomized animals.

5. Thyroparathyroidectomized rabbits develop anti-sheep hemolysin of a uniformly low titer—on an average one-fifth that of the controls.

6. Injection of bovine blood into rabbits that survived complete thyroparathyroidectomy from 1 to 2 months previously results in the

production of hemolysin of a uniformly low titer compared with that of normal animals similarly treated.

We owe our sincere thanks to Dr. H. T. Karsner for advice and aid during the progress of this work, and to Dr. Julius M. Rogoff and Dr. J. Lucien Morris for technical assistance.

BIBLIOGRAPHY.

Carlson, A. J., and Woelfel, A., *Am. J. Physiol.*, 1910, xxvi, 32.
Farner, E., and Klinger, R., *Mitt. Grenzgeb. Med. u. Chir.*, 1920, xxxii, 353.
Fassin, L., *Compt. rend. Soc. biol.*, 1907, lxii, 388.
Fassin, L., *Compt. rend. Soc. biol.*, 1907, lxii, 467.
Ferreira de Mira, M., *J. physiol. et path. gên.*, 1920, xviii, 976.
Fjeldstad, C. A., *Am. J. Physiol.*, 1910, xxvi, 72.
Frouin, A., *Compt. rend. Soc. biol.*, 1910, lxix, 237.
Garibaldi, A., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 15.
Gates, F. L., *J. Exp. Med.*, 1918, xxvii, 725.
Hagenbach, E., *Mitt. Grenzeb. Med. u. Chir.*, 1908, xviii, 329.
Hastings, A. B., and Murray, C. D., *J. Biol. Chem.*, 1921, xlvi, 233.
Hektoen, L., *Tr. Chicago Path. Soc.*, 1911, viii, 138.
Hektoen and Carlson, quoted by Fjeldstad.
Hektoen, L., and Curtis, A. C., *J. Infect. Dis.*, 1915, xvii, 409.
Houssay, B. A., and Hug, E., *Rev. Inst. bact., Dept. nac. hig.*, 1920, ii, 637.
Koopman, J., *Endocrinol.*, 1919, iii, 318.
Launoy, L., and Lévy-Bruhl, M., *Compt. rend. Soc. biol.*, 1913, lxxv, 352.
Launoy, L., and Lévy-Bruhl, M., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 90.
Launoy, L., and Lévy-Bruhl, M., *Ann. Inst. Pasteur*, 1915, xxix, 213.
Lerda, G., and Diez, S., *Gior. r. Accad. med. Torino*, 1905, lxviii, 195.
Loevenhart, A. S., and Grove, W. E., *J. Pharmacol. and Exp. Therap.*, 1910, i, 289.
Marbé, S., *Compt. rend. Soc. biol.*, 1908, lxiv, 1058.
Marbé, S., *Compt. rend. Soc. biol.*, 1908, lxiv, 1113.
Marbé, S., *Compt. rend. Soc. biol.*, 1909, lxv, 612.
Marbé, S., *Compt. rend. Soc. biol.*, 1909, lxvi, 1073.

EXPLANATION OF PLATES.

PLATE 19.

FIG. 1. Low power photomicrograph showing parathyroid gland (*a*) situated outside of capsule of thyroid gland (*b*).

PLATE 20.

FIG. 2. Low power photomicrograph showing parathyroid gland (*a*) situated within the parenchyma of the lateral lobe of the thyroid gland (*b*).

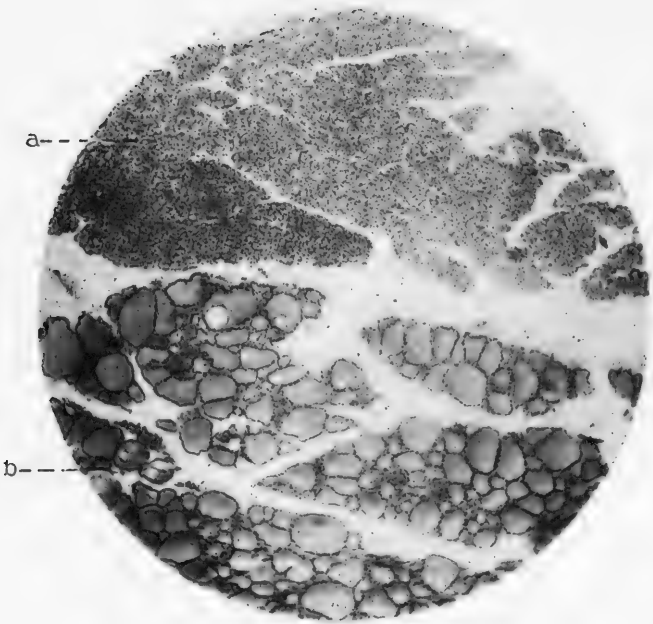


FIG. 1.

(Ecker and Goldblatt: Thyroidectomy and parathyroidectomy.)

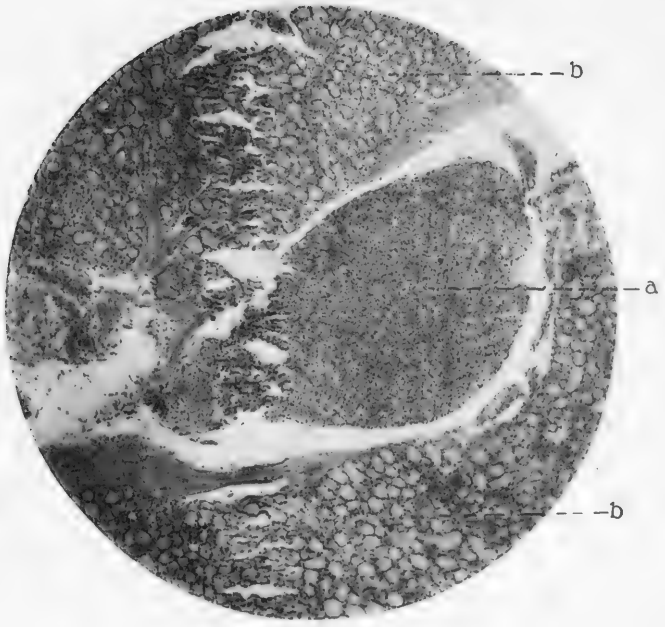


FIG. 2.

(Ecker and Goldblatt: Thyroidectomy and parathyroidectomy.)

CRISTISPIRA IN NORTH AMERICAN SHELLFISH. A NOTE ON A SPIRILLUM FOUND IN OYSTERS.

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PLATES 21 TO 24.

(Received for publication, April 1, 1921.)

In the European lamellibranchiata there have been found a large number of comparatively coarse forms of spiral organisms, provided with a broad, band-like membrane extending from one extremity to the other and winding itself obliquely around the body, one edge being attached to the body, the other free. The organism was first discovered in oysters by Certes (1), who regarded it as akin to the trypanosome and called it *Trypanosoma balbianii*. Laveran and Mesnil (2) considered it a bacterium, while Perrin (3), working under Schaudinn, thought it a protozoon. At that time Schaudinn (4) held the view that the spirochete represented a phase of the trypanosome and that both belonged to the protozoa. The peculiar structure of the organism, as brought out by the technique employed by Perrin, suggested the mitotic and other nuclear figures to such an extent that the organism was accepted as a protozoon by certain protozoologists (5–8). The investigations by Swellengrebel (9) and later by Gross (10) led the latter to place it in the class of *Cristispira*, a terminology accepted by Dobell (11) and Zuelzer (12). The points in dispute are: (1) the interpretation of the membrane; (2) the presence or absence of a periplast; and (3) the presence or absence of a nuclear apparatus, especially the significance of the chambered structure of the body. These questions, which had never been definitely settled, led the writer to undertake examinations of American shellfish. Furthermore, so many species have been described recently in the European shellfish that a comparison from the standpoint of geographic distribution of these cristispiras seemed desirable.

TABLE I.

Spirochæta (*Schellack*).

Name.	Source.	Length.		Breadth.		Ends.
		Average.	Extremes.	Average.	Extremes.	
Spirochæta balbianii.	*Ostrea edulis.*	39μ	35–42μ	1.3μ	1.1–1.5μ	Rounded, no terminal appendage.
" *ostreæ.*	" "	41.5μ	38–42.5μ	1.1μ	1.0–1.3μ	Sharp, " " "
" *chamæ.*	*Chama gryphoides.* " *sinistrorsa.*	45.6μ	45–46.5μ	1.4μ	1.3–1.5μ	Rounded, " " "
" *anodontæ.*	*Anodonta mutabilis.*	46μ	39–50.5μ	1.0μ	0.9–1.2μ	" " " "
" *spiculifera.*	"	33μ	28–36.5μ	0.9μ	0.7–1.1μ	Pointed, terminal filament.
Spirochæta modiolæ.	*M. barbata.*	37.5μ	36–40μ	0.8μ	0.7–0.9μ	Rounded, no terminal appendage.
" *pinnæ.*	*P. nobilis.*	30.4μ	29–31μ	1.0μ	0.8–1.1μ	" " " "
" *limæ.*	*L. inflata, L. hians.*	37μ	35–41μ	1.4μ	1.0–1.8μ	" " " "
" *cardii papillos.*	*C. papillosum.*	19.1μ	18.5–20μ	1.2μ	1.1–1.4μ	" " " "
Spirochæta tapetos.	*T. decussata.*	34.5μ	29–35μ	1.3μ	1.1–1.4μ	Rounded, occasionally terminal appendage.
" *acuminata.*	*Tapes læta.*	47μ	43.5–49.5μ	1.0μ	0.9–1.1μ	Pointed, no terminal appendage.
Spirochæta saxicavæ.	*Sax. arctica.*	31μ	30–32μ	1.7μ	1.6–1.8μ	Rounded, " " "
" *gastrochænæ.*	*G. dubia.*	29μ	Constant.	1.2μ	1.1–1.3μ	One end blunt, one sharp, no terminal appendage.
*Spirochæta pusilla.**	*Anodonta, Unio, Lima, Tapes, etc.*	13μ	12–14μ		0.3–0.4μ	Sharp, pointed.

* Bosanquet found a spirochete 10 to 12 μ in length which he thinks may be identical with *Spirochæta hartmanni* of Gonder or with *Spirochæta pusilla* of Schellack. (No crista?)

Schellack (13), Keysselitz (5), and Gross (10, 14) created a large number of species with only slight morphological variations. To quote an example, Schellack set up thirteen species with certain characteristics, as shown in Table I.

As will be pointed out later, the method of classifying these coarse organisms by their morphological features is subject to error because of the great variations in the terminal portions and in the thickness of body according to the degree of fixation, dehydration, or staining; a difference in these factors may cause the same organism to assume a totally different aspect. Hence the writer has studied these organisms not only in stained preparations but also under the dark-field microscope.

TABLE II.

Occurrence of Cristispira in the Styles of North American Shellfish.

Shellfish.	No. of specimens examined.	Styles present.	*Cristispira* present.
Ostrea virginiana	298	128	99 (40 degenerated).
Venus mercenaria	110	70	8 (3 ").
Modiola modiolus	97	73	4
Ensis americana	24	20	0
Mya arenaria	24	24 (hard).	0
Mactra solidissima	12 (dead?).	1	0
Mytilus edulis	42	34	0
Pecten irradians	39	34 (hard).	0
Fulgur canaliculatus	8	2 (?)	0
Nassa obsoleta	30	4	0

The present study deals only with salt water shellfish caught in the neighborhood of Woods Hole during the month of August, 1916, and includes *Ostrea virginiana* (oysters), *Venus mercenaria* (quahaugs), *Mya arenaria* (long neck clams), *Ensis americana* (razor clams), *Mactra solidissima* (large sea clams), *Pecten irradians* (scallops), *Mytilus edulis* (mussels), *Modiola modiolus* (mussels), *Fulgur canaliculatus* (winkles, whelks), and *Nassa obsoleta*. The number of specimens of each species examined and the positive findings of *Cristispira* and styles are shown in Table II.

As the tabulation shows, *Cristispira* was found most frequently in the styles of oysters, next in clams, and then in modiolas. No other species examined showed any *Cristispira*. In handling the

styles of these shellfish it was observed that those of oysters, although moderately solid, became liquefied into a viscid fluid upon standing for half an hour at room temperature in a small amount of sea water after removal. The styles of modiolas are much smaller than those of oysters and somewhat firmer; those of clams are much more solid and elastic than either of the others and did not become liquefied on exposure to air and sea water at room temperature. Scallops have large and firm styles which remain little changed after removal. Styles from the other varieties are very small and solid. The physical properties of the styles may be partly or chiefly responsible for the presence or absence of *Cristispiræ* in these shellfish; apparently the consistency of the styles of oysters offers the optimum conditions for the habitation of the organism.

Another striking fact is that, at least in the case of the oysters, the longer they were kept in a tank (eel pond) or the car (sea) after collection from their original beds, the less frequently they contained styles. In such specimens the presence of *Cristispira* is also less frequent, and in many of them only degenerated forms may be found. It is rather easy to determine the freshness of oysters by examining them for styles and the activity of the cristispira contained in them; even freshly collected oysters soon lose the styles if they are not extracted immediately. In one instance 48 oysters which had been kept in the eel pond 24 hours after collection were opened one after another and left at room temperature for about 30 minutes, when they were searched for styles, but unsuccessfully. Six more oysters from the same lot which had not been opened all had perfect styles, and five contained *Cristispira*. These external factors must be taken into consideration in making a survey as to the frequency of *Cristispira* infection of oysters. This rule does not apply to those shellfish whose styles are firm and resistant to manipulation.

The main object of the present work was to study the conditions under which these coarse spiral organisms can be kept alive or cultivated on artificial media, in order that their biological and morphological characteristics might be better studied. Experiments were also made in which the organisms were subjected to the action of various salts, acids, and alkalies in order to obtain an insight into the finer

structure of the organism. Vital stainings with neutral red, crystal violet, brilliant cresyl blue, methyl green, methyl orange, Bismarck brown, and Janus green were also applied.

Dark-Field Examination of Styles.

Oysters.—The style from the oyster is a glassy, somewhat elastic, cylindrical body of about 2 to 3 cm. long and about 3 mm. in diameter at the thickest portion, tapering to a point at the end. When placed in a Petri dish it becomes a sticky viscid fluid; it dissolves in sea water. When a drop of the liquefied style is examined under the dark-field microscope there are observed in many instances violently motile organisms of large size moving through the field with great velocity. They pass out of the field so rapidly that their exact form is not at once recognizable; they suggest butterflies constantly flapping luminous wings; their movements are backward as well as forward. They become sluggish within a short time and come to rest either in a tortuous form or stretched out. The body is a long cylinder with blunt ends. The end portion is slightly thinner than the rest, but not sharply drawn. There is a thick refractive margin all along the body, indicating the presence of definite peripheral concentration of the protoplasma (perhaps a cell membrane). The inner part of the body seems to present no distinct transverse partitions (so called chambered structure), but there are rows of refractive projections from both sides of the wall, point to point, as if they were imperfect cross-bars; the intervals between the bars are about 3 μ. Along the entire body runs a peculiar undulatory narrow membranous structure coiling obliquely and spirally, showing a free edge here and there (known as the crista, or ridge, of the cristispira). It follows a wavy course, and at each wave summit the light is highly refracted. From that point innumerable fibrillar, fan-like radiations run into the body of the organism upon which the other edge of the membranous structure rests or is in connection (Figs. 1 to 3). The fibrils along the free edge of the crista seem to be so arranged as to form a more compact structure than the portion which stretches between the edge and body of the organism. Locomotion appears to be accomplished by alternate bending and relaxation of the undulatory, fibrillar, highly elastic membrane, as is easily demonstrated in a specimen whose

motility is slackening because of exhaustion. In a later period of exhaustion the organism becomes immotile, and the elastic wavy frame of the membrane assumes its full length and shape, causing the body to conform to its spirals (Fig. 8). Only active specimens take the shape of a snake (Figs. 1 to 3). In a freshly extracted style which is still unsoftened by exposure to the air we often find a nest of organisms in one field (Figs. 4 and 5), some assuming positions like coiled snakes, and some rather regularly waved and stretched out. In a few moments the former move rapidly from their resting places with a violent locomotion and swim away; the latter, which represent skeletons or elastic frames (Fig. 8), are degenerated and remain immotile. When a liquefied style containing active specimens of the organism is left for several hours under unfavorable conditions (room) most of the organisms undergo degeneration and are found in various stages from merely stretched out, regularly wavy organisms to skeletons without the body (Figs. 6, 7, and 9). The disintegration of the body is shown by an irregular contour and various degrees of thickness of the body substance. The skeleton often shows clearly its component elastic fibrils, some of which may take the shape of a bushy horse tail (Fig. 6). The gradual overpowering of the body by the elastic membrane in a dying specimen is shown in Figs. 7 and 9.

Clams.—The styles of clams are thicker, firmer, and somewhat longer than those of oysters, faintly yellowish, shiny, somewhat opaque, and do not undergo liquefaction in sea water when removed to a Petri dish. For dark-field examination it is necessary to macerate them in a mortar in order to obtain an emulsion. Five only, of 110 styles examined, showed the presence of active *Cristispiræ*; 3 others contained degenerated specimens. The dark-field appearance of the cristispiras found in clams was identical with that of the cristispiras in oysters (Fig. 10).

Modiolas.—The modiolas are small mussels, with proportionately small vitreous styles, not longer than 1 cm.; the consistency of the styles is softer than that of clams but firmer than that of oysters. For dark-field examination they can be easily macerated on the slide by pressing under a cover-glass. *Cristispiræ* were present in 4 of 97 styles studied. The organism showed microscopically no distinctive features except that of exceptional length (Fig. 11).

Examination by Means of Vital Staining.

The cristispiras from oysters were used for this purpose.

Neutral Red.—Within a few minutes the body showed a general brownish yellow coloration, with numerous paired yellowish granules scattered with fair regularity along the entire wall. The membrane, or crista, appeared as bushy brownish fibrils projecting in all directions. The color of the body disappeared in 20 minutes, that of the fibrils in 6 hours. Coiled specimens did not take the stain.

Crystal Violet.—The body took a rather deep purple stain, the crista remaining uncolored. Fine protoplasmic reticula with intersecting granules were seen. In some specimens vacuolation of the cell contents occurred, a large mass of substance occasionally protruding into the side (plasmolysis). The deep purple color was still present at the end of 6 hours. There was no chambered appearance of the body.

Brilliant Cresyl Blue.—The body took up in mottled fashion a light bluish lavender color. The cross-bars (bluish) of the body were distinctly brought out in a few minutes; the crista remained practically unstained. Complete plasmolysis and decoloration occurred within 6 hours.

Methyl Green.—The body stained faint pink without showing any structure; the crista was not stained. Decoloration occurred in 6 hours.

Methyl Orange.—There was no coloration. The organisms remained active.

Bismarck Brown.—The body became distinctly brownish, with more deeply stained granules throughout the entire length. These granules sometimes occurred in a line along one or both sides of the wall. Degenerated specimens were not well stained. The specimens were practically decolorized in 6 hours.

Janus Green.—The body took a deep, dark bluish color, with reticular mottles, the crista a dark blue on its free margin but only a faint blue elsewhere. In some specimens comparatively large round granules of varying size, stained dark bluish, were seen scattered about in several parts of the body. Degenerated organisms did not take the stain. Within 6 hours the color gradually faded.

In general, then, the body of *Cristispira balbianii* took the vital staining, while the crista stained only slightly with some of the dyes and not at all with others. The phenomenon common to staining with neutral red, brilliant cresyl blue, Bismarck brown, and Janus green was the fact that within the first 5 minutes the body of the organism took the stains in such a way as to show the presence of chromophil granules more or less regularly and diametrically paired on both sides of the wall. After a longer period the number of chromophil granules became more numerous, and a sort of reticular distribution of the same substance was brought out, giving the entire body a mottled appearance. In the case of brilliant cresyl blue the cross-bar arrangement of the chromophil substance was distinct but this was followed by complete plasmolysis of the organism, indicating that the chambers were the result of an unfavorable or toxic effect of the dye. Coiled specimens usually showed no deeply staining granules with Bismarck brown or neutral red.

Stained Preparations.

Several methods were used for the study of the morphological characteristics of the cristispiras, but the best differentiation of the various constituents of the organism was obtained by application of Giemsa's stain and Heidenhain's iron-hematoxylin.

With Giemsa's stain the organisms were variously fixed. In some instances the liquefied styles containing the cristispiras were thinly spread over a clean slide and the moist film surface was immediately exposed to osmic acid vapor (1 per cent) for 1 minute. 30 minutes fixation in absolute alcohol followed. In other instances the moist films were at once immersed in a jar containing Schaudinn's sublimate alcohol, which was maintained at a temperature of 75°C. for 5 minutes and then allowed to cool. Before applying Giemsa's solution the films were rinsed in water, treated first with Lugol's solution, then with 0.5 per cent sodium thiosulfate solution, then thoroughly washed in water. In this procedure the films were never allowed to dry until after being stained with Giemsa's solution. In still other instances the films were first dried in the air, then fixed in methyl alcohol for 30 minutes, then stained with Giemsa's solution.

The object of these various procedures was to study the influence of fixation on the morphology of the organism.

The morphological features revealed by Giemsa's stain were very similar, whether the films were fixed by the osmic vapor or by Schaudinn's hot sublimate alcohol method. In both preparations the body showed a cross-bar structure consisting of deeply stained blue bars alternating with light, almost unstained or faintly pinkish areas. The bars, which were correspondingly wider in larger specimens, appeared somewhat narrower toward the extremities. The relative width of the bluish bar and of the juxtaposed light spaces was very variable in different specimens. In some the width was nearly the same (Figs. 29, 30, and 40); in others the light spaces were much wider than the cross-bars, which then appeared like thin cross lines (Figs. 12, 13, and 31); in still others the reverse was true, in which case a deeply stained bluish body seemed to be segmented by narrow light cross spaces (Figs. 14, 15, 17, and 32 to 34). In the specimens fixed in sublimate alcohol there were specimens showing in some portion of the body several consecutive bars, so thinned out near the the middle as to be almost broken (Figs. 13, 17, and 35), the appearance being not unlike that of the organism in the fresh state. The bluish cross-bars were not of uniform width or sharply defined borders, but one end was usually broader than the other, and sometimes a few here and there were placed obliquely to the right angle formed by the wall and the hypothetical axis of the body. In contrast to fresh specimens, the stained organism generally had a tapering form, ending in sharply pointed extremities (Figs. 12 to 14, and 29 to 35). The crista took a brilliant red hue, showing one or more heavily stained marginal fibers, and it ended near the end of the body (Figs. 29 to 35). The connection between the body and the heavy marginal fibers of the crista is so thin that the finer fibrillar structure is seldom brought out. One may encounter in stained preparations specimens caught in full activity (Figs. 12, 14, 15, 17, 19, 29 to 33, and 35) or in the exhausted condition, yielding to the elastic crista, with beginning plasmolysis (Figs. 20 and 21) or with the crista skeleton (Figs. 23 and 36). Sometimes the crista alone may be found (Figs. 37 to 39).

The specimens fixed in methyl alcohol after being air-dried did not show so sharp a structure as those just described. The organism

stained more reddish in general, and the bluish bars were not clearly distinguished (Figs. 16, 18, and 19).

Heidenhain's iron-hematoxylin stain was applied to film preparations of the cristispira derived from clams and Mallory's to sections of oyster styles. In the latter the organisms were present in large numbers, usually lying parallel with the course of protein lamellæ, which run concentrically along the long axis of the style like a scallion. Some were coiled, but most assumed wavy forms (Fig. 22). The cross-bar structure was easily distinguished. The crista is occasionally revealed—it is perhaps held closely to the body. (In fresh specimens the crista can always be seen.) Occasionally there are found specimens cut across so that they are visible as a ring. The details of structure are more clearly brought out in the film preparations by adequate differentiation (Figs. 25, 27, and 44). The body is cross-barred by thin, dark grayish lines, with a more definite contour of the cell wall. The crista is fibrillar and retains a light bluish gray color which is deeper along the marginal fibers. The extremities of the body are not sharply drawn out but are blunt points.

In one of the clams collected in New York Bay in September, 1916, I encountered a rather long variety of *Cristispira*. It was similar in all other respects to the variety met with in the clams studied at Woods Hole. Whether this specimen of *Cristispira* is a new species or merely a variety due to temporary factors has not been determined (Figs. 24, 26, 28, 45, and 46).

Effects of Chemicals.

The microchemical reactions of various components of *Cristispira balbianii* were studied with a view to gaining an insight into the structure of the organism. Previous investigators have obtained varying results in a similar study of the European specimens. The writer's studies with the American variety were made with the following substances: sodium taurocholate, sodium glycocholate, sodium oleate, saponin, cobra lecithid, acetic acid, hydrochloric acid, sulfuric acid, potassium hydroxide, and ammonia.

Method of Study.—One part of the style emulsion containing numerous active specimens of *Cristispira balbianii* was mixed on a coverglass with one part of the substance, dissolved in sterile sea water;

TABLE III.

Effect of Chemicals on Cristispira.

Date.	Chemical.	Concentration.	Result.
1916		*per cent*	
Aug. 23	Sodium taurocholate.	10	Plasmolysis complete in 5 to 10 min. Skeleton, or crista, resistant. *Spirillum ostreæ* dissolved; bacilli still motile. After 20 hrs. at room temperature skeletons (cristas) showing their fine fibrillar composition are more completely denuded of the cell plasma.
		1	Practically the same as with 10 per cent.
		0.1	Nearly all have undergone plasmolysis; otherwise the same as preceding experiment.
	Sodium glycocholate.	10 1 0.1	Similar in effect to the taurocholate solution.
	Sodium oleate.	10 1 0.1	This salt produced such a viscid emulsion that no observation was possible.
	Cobra lecithid.		Almost insoluble in sea water. Observations indecisive.
	Saponin.	10	Complete plasmolysis in 5 to 10 min. Crista not affected but assumes more regularly waved spiral bundles of fibrils (Fig. 9), to which irregularly protruding masses of protoplasm are seen to be attached at some points. *Spirillum ostreæ* and bacilli still motile. After 20 hrs. at room temperature the cristas are still intact. They show their finer structure and their relation to the bodies, which are now mere masses of broken up protoplasm scattered along the regularly undulated spiral cristas.
		1	Same as with 10 per cent.
		0.1	Nearly all have undergone plasmolysis.
	Controls in sea water.		Actively motile for 2 hrs. after being sealed on slides; thereafter gradually became immotile; structure unchanged. After 20 hrs. no degeneration but all in "relaxed" state.

TABLE III—*Concluded.*

Date.	Chemical.	Concentration.	Result.
1916		*per cent*	
Aug. 24	Acetic acid.	50	After 2 hrs. body smooth, showing cross-bar structure and heavy cell wall; crista shrivelled and indistinct; *Spirillum ostreæ* swollen and indistinct.
		10	Same as with 50 per cent.
		1	" save for the presence of many round bodies suggesting extruded protoplasm of the body of *Cristispira balbianii.*
	Hydrochloric acid.	50	After 2 hrs. body shows more densely set cross-bar appearance than in acetic acid, but the crista, though perhaps thickened and less distinct than normally, is still attached to the body.
		10	Same as with 50 per cent.
		1	" except for some nodular swelling of the body in parts.
	Sulfuric acid.	50	Similar to results with hydrochloric acid except for more granular appearance of the body substance and less distinctness of the crista.
	Potassium hydroxide.	15	Within 2 hrs. the organisms have practically disappeared, except for a few fragments of the bodies in which no structure save the cell wall can be recognized; no crista found.
		3	Within 2 hrs. the body shows irregularly contoured thick wall with vestiges of thinned out, broken cross-bars; crista seems to be closely attached to the body.
		0.3	Within 2 hrs. there are apparently no changes; body shows distinct cross-bars and heavy cell wall; crista seems to be intact.
	Liquor ammoniæ fortis.	50	Within 2 hrs. no changes; immobilized.
		10	" 2 " " " "
		1	" 2 " " " "
	Controls in sea water.		After 2 hrs. almost all are still active.

the cover-glass was then sealed with vaseline on a slide and examination made under the dark-field microscope. The concentration of each substance and the effects of each upon the organism are briefly summarized in Table III. The observations were made at room temperature (23°C.).

Survival of Cristispira balbianii under Different Conditions.

An oyster emulsion containing actively motile cristispiras was used to study the effects of tonicity. The observations were made in hanging drop preparations after 2 hours at room temperature (22°C.). When mixed with distilled water the organisms became immotile, then the cell body assumed a regularly spiral course in conformity with the skeletal crista, presenting at the same time accumulations of a highly refractive substance in round or oval masses along the body at each turn of the spiral skeleton. The phenomenon may be interpreted as due to plasmolysis through hypotonicity. On the other hand, in 10 per cent sodium chloride solution the organisms remained intact, but many coiled forms (Fig. 40) were seen. The body appeared less refractive, but there was no plasmoptysis. In the control preparations made with sea water, most of the organisms became immotile after 2 hours at room temperature; in those kept at 6–8°C. all the cristispiras were still actively motile at the end of 2 hours, and one-fifth to one-tenth after 24 hours; after 40 hours only a few were found to be active in one of the three slides. In dark-field preparations (not hanging drop method), sealed with paraffin and kept at 6–8°C., all cristispiras were disintegrated at the end of 24 hours.

Attempts at Cultivation.

Several unsuccessful attempts were made to obtain a culture of *Cristispira balbianii*. The chief obstacles in the work were (1) the lack of bacteriological facilities, and (2) the impossibility of obtaining a bacteria-free suspension of *Cristispira balbianii*.

The culture media employed in the first attempts were made with a filtrate of various styles (oysters and scallops), obtained by passing the sea water solution (or emulsion) of styles (50 styles in 100 cc. of sea water) through a Berkefeld filter V. To 3 cc. of the filtrate were

added, in one set, 1 cc. of 2 per cent glucose agar; in another set 1 cc. of 2 per cent plain agar; in a third set 1 cc. of ascitic fluid; in the fourth 1 cc. of glucose broth; in the fifth 1 cc. of sterile sea water; and in the sixth (controls) 4 cc. of sterile sea water without the style filtrate. All tubes were covered with a thin layer of sterile paraffin oil to prevent evaporation of the culture media. The cultures were set up as follows: All tubes (1 cm. in diameter and 22 cm. high) first received 0.2 cc. of a rich cristispira emulsion from oysters. 3 cc. of the style filtrate were then added to all except the control (sixth) set. The first and second sets were next mixed with 1 cc. of 2 per cent melted glucose agar and plain agar, respectively, the temperature of the agar being about 42°C. at the time of mixing. The tubes were cooled quickly by immersion in the aquarium. The glucose broth and ascitic fluid were added to the other sets and the paraffin oil was finally added to all. Each set was made up in duplicate, one being kept at room temperature and the other at aquarium temperature (about 7°C.).

Examination of the culture tubes was made daily during 8 days, but no growth of cristispira was ascertained. They were brought to The Rockefeller Institute in an ice-packed container and followed for 3 weeks longer, but except in one instance, in which a peculiar large non-motile organism was seen to grow, the results were negative. This organism, which grew in the first set of media (semisolid), bore a close resemblance to *Cristispira*, except that there was no crista. It suggested Gross' *Saprospira*. Whether it was a cristispira modified through artificial cultivation (loss of crista) or an altogether different organism is still a question. Most of the tubes were more or less contaminated with members of the *mesentericus* group.

A spiral organism of oysters, *Spirillum ostreæ, n. sp.* (Fig. 43), likewise failed to grow. This spiral organism measures about 8 to 16 μ in length and 0.5 μ in the widest portion of the body. Both ends gradually taper off to fine filaments. The number of spirals varies from 4 to 8 and the curves are rather shallow but very regular. The body is elastic but not perceptibly flexible. They rotate rapidly but seldom proceed one way or the other.

In another experiment styles were ground in a mortar with Ringer's or Locke's solutions as well as with sea water and the whole was

sterilized in an autoclave. The opalescent fluids thus obtained were used in experiments similar to those preceding with unsatisfactory results.

The tissue culture technique was employed in order to determine the viability of cristispiras in the presence of various extraneous substances. By careful examination it was possible to collect a dozen styles rich in cristispiras and apparently free from ordinary bacteria. *Spirillum ostreæ* was the only variety present and occurred in rather large numbers in some styles. The styles were dissolved in 3 cc. of sterile Ringer's solution; the work was carried out as aseptically as circumstances permitted.

To 1 drop of the style emulsion was added 1 drop of the following liquids respectively: (1) mixture of oyster style-Locke's solution infusion filtrate; (2) oyster broth-Locke's solution-infusion-style filtrate; (3) oyster juice filtrate; (4) oyster broth; (5) Locke's solution; (6) sea water; (7) distilled water; (8) ascitic fluid; and (9) bouillon. Eight Ringer's solution preparations were made as controls.

At the end of 24 hours at 6–8°C. many *balbianii* remained active in three of the eight control (Ringer's solution) hanging drop slides, and in the slide containing ascitic fluid. In all other instances the organisms were inactive and diminished in numbers; there was an increasing mass of bacteria more marked in the slides containing albuminous solutions like oyster-filtrate, Locke's solution-style infusion, and bouillon. At the end of 48 hours only a few cristispiras were still sluggishly motile in the slides containing ascitic fluid and in one of the Ringer's solution controls.

This observation serves to emphasize the difficulty of obtaining a culture of this organism under the conditions of the experiments just described.

DISCUSSION AND SUMMARY.

Ten varieties of North American shellfish were examined for the occurrence of *Cristispira* in their styles. A cristispira was found in various numbers in *Ostrea virginiana*, *Venus mercenaria*, and *Modiola modiolus*, but none in *Ensis americana*, *Mya arenaria*, *Mactra solidissima*, *Pecten irradians*, *Mytilus edulis*, *Fulgur canaliculatus*, or *Nassa obsoleta*. Of 298 oysters, only 128 showed the crystalline styles, in

which cristispiras were present in 99. Active cristispiras were found in 59 styles only and degenerated forms in the remaining 40. In 110 clams (*Venus mercenaria*) 70 styles were found, and only 8 of these contained cristispiras; 5 yielded active and the other 3 degenerated cristispiras. In 97 modiolas there were 73 styles, only 4 of which contained cristispiras.

The physical properties of the crystalline styles of these shellfish varied considerably. The styles of the oysters were moderately soft, and when exposed to the air or mixed with sea water they underwent liquefaction, forming a clear, viscid material. The styles from clams and modiolas were opaque and were more firm, not easily crushed even in a mortar. The styles of the scallops were the most solid of all the styles examined. It happened that the softer the styles, the more frequent was the occurrence of the cristispira; in fact, no cristispira was detected in styles other than those of oysters, clams, and modiolas, of which oysters had the softest styles and the largest percentage of cristispira invasion.

The following observations were made regarding the structure of the cristispira found in oysters. The body is a long, flexible cylinder, with blunt extremities, towards which the diameter gradually diminishes. In motion the body rapidly stretches and contracts, forming in the contracted state several serpentine undulations. A membranous appendage (Gross' crista) winds about the body throughout its entire length. The inner margin is in connection with the body, the outer margin is free and is distinctly heavier. The latter is undulatory; that is, the width of the membrane, or crista, is narrower at some points than at others. The membrane is composed of numerous fine fibrils running in a roughly parallel or slightly oblique course, showing interwoven narrow meshes; at the outer margin there is a dense smooth ridge.

The contour of the body is highly refractive, as if possessing a cell membrane. The interior structure, as revealed by dark-field illumination, is an almost homogeneous, less refractive substance, but there are present minute highly refractive granules more or less symmetrically arranged. There is no definite cross-bar or chambered structure. On the other hand, when vital staining with brilliant cresyl blue is applied, there appear numerous paired masses of lavender hue at

fairly regular intervals, suggesting the cross-bar aspect of a stained specimen. In a few specimens there was seen a dim outline of cross-bar effect. Neutral red, Bismarck brown, and crystal violet all bring out deeply stained granules and reticular structure but no definite cross-bars.

While it is difficult to recognize any definite chambered structure in the fresh state, it is very easy to bring out the characteristic banded body by staining the organism with Giemsa's solution. The bands, or cross-bars, which take up a deep bluish color, are of variable width, ranging from a thin line to a square or even oblong block. In some specimens the body is almost blue with thin white cross lines, while in others the blank body is banded with thin bluish bands at irregular intervals. The crista, or undulatory membrane, is stained bright red, particularly along the outer free margin. The fibrillar structure of the crista is usually recognizable. In the specimens stained with Heidenhain's iron-hematoxylin the cross bands, which appear bluish gray, are more distinct and delicate. The bands are not, however, always thin and sharp, but in certain specimens are almost square. The irregularity in the width of the cross bands in various parts of one specimen and in different specimens suggests that the peculiar banded appearance may be a result of fixation and not the natural structure of the organism. At least, no such structure was recognized under the dark-field microscope, which would have revealed such definite septa if they existed. I believe that the cross bands are formed from a homogeneous mass through sudden contraction due to dehydration with absolute alcohol during fixation. In the preparations first dried in the air and then fixed with methyl alcohol this banded appearance is absent. That *Cristispira* has a chambered body, each chamber being equivalent to a single cell, is Gross' interpretation, which is not supported by the observations reported here. Moreover, the protoplasm of the organism readily escapes from the body in distilled water, saponin, or other chemical agents, forming many round masses of extruded protoplasm in different parts of the body. No septa are to be seen in these plasmolyzed or degenerated organisms. Acids, hydrochloric and acetic, failed to bring out any banded structure of the body. It may be deduced that the external surface of the body is a highly refractive cell membrane, and that

within the cell body are homogeneous protoplasm of reticular form and numerous highly refractive granules of varying sizes. Some of the contents take the dark bluish color when stained with Giemsa's solution. In a fixed specimen this particular substance becomes contracted into a series of masses of varying dimension, leaving between each mass a blank space of varying width, and giving a characteristic cross-barred or banded (so called chambered) structure. In some specimens the cross-bars are not seen, but instead a series of broken lines running parallel with the longitudinal axis of the body (Figs. 41 and 42).

The crista is a fibrillar structure, connected with the body at its inner edge. The outer margin is a thickened bundle of fibrils running an undulating course along the entire length of the crista. The crista is elastic and when detached from degenerated organisms assumes a rather regularly wound spiral, consisting of longitudinal bundles of fibrils (Figs. 36 to 38). A fragment of two or three waves may be encountered in a preparation containing many degenerated organisms (Fig. 39). The composition of the crista can best be studied in degenerated remains of the organism. During the life of *Cristispira* it is stretched or relaxed according to the contraction or extension of the body. The elasticity of the crista appears to furnish the organism with a propelling and rotating power upon its extension after being drawn tightly to the body by some contractile apparatus (myoneme) present somewhere within the cell body. The crista serves as a rudder and propeller for the swimming organism. It is interesting to compare here the elastic and regularly waved flagella of certain bacteria and spirochetes; it is possible that the crista of *Cristispira* is a highly modified form of flagella.

The nature of the substance which stains dark blue with Giemsa's stain is not known, but it does not give a chromatin reaction. By Heidenhain's iron-hematoxylin method it takes a dark grayish tint, similar to the cell wall or crista, which are also dark gray. This substance was regarded by Gross and Zuelzer as volutin, which is of nutritive origin. It is probable that there are also embedded within it minute chromidial elements. Multiplication is by transverse fission.

Cristispira balbianii is parasitic and does not survive more than a few days in ordinary sea water emulsion, even at its optimum

temperature. In its natural habitat, or the crystalline style, it is usually pure, but is sometimes found in association with a tiny spiral organism (*Spirillum ostreæ*). The cristispiras in the styles seem to diminish rapidly when oysters are collected from their beds and transferred elsewhere; oysters kept in tanks or cars for several days do not contain the cristispiras, and in opened oysters the styles disappear promptly at room temperature.

All efforts to cultivate this organism have failed.

BIBLIOGRAPHY.

1. Certes, A., Les parasites et les commenseaux de l'huitre, *Bull. Soc. zool. France*, 1882, vii, 347; Sur le *Trypanosoma balbianii*, 1891, xvi, 95.
2. Laveran, A., and Mesnil, F., Sur la nature bactérienne du prétendu Trypanosome des Huitres (*Tryp. Balbianii* Certes), *Compt. rend. Soc. biol.*, 1901, liii, 883.
3. Perrin, W. S., Researches upon the life history of *Trypanosoma balbianii* (Certes), *Arch. Protistenk.*, 1906, vii, 131.
4. Schaudinn, F., Generations- und Wirtswechsel bei *Trypanosoma* und *Spirochæte*, *Arb. k. Gsndhtsamte*, 1904, xx, 387.
5. Keysselitz, G., Beschreibung von *Spirochæta anodontæ nov. spec.*, *Arb. k. Gsndhtsamte*, 1906, xxiii, 566.
6. Keysselitz, G., Studien über Protozoen, *Arch. Protistenk.*, 1908, xi, 334.
7. Höing, A., Vergleichende Untersuchungen über Spirochäten und Spirillen, *Arch. Protistenk.*, 1911, xxiii, 101.
8. Fantham, H. B., *Spirochæta* (*Trypanosoma*) *balbianii*, (Certes) and *Spirochæta anodontæ* (Keysselitz): their movements, structure, and affinities, *Quart. J. Micr. Sc.*, 1908, lii, 1.
9. Swellengrebel, N. H., Sur la cytologie comparée des spirochètes et des spirilles, *Ann. Inst. Pasteur*, 1907, xxi, 448; *Compt. rend. Soc. biol.*, 1907, lxii, 213.
10. Gross, J., *Cristispira nov. gen.*, ein Beitrag zur Spirochætenfrage, *Mitt. zool. Station Neapel*, 1910, xx, 1.
11. Dobell, C. C., On *Cristispira veneris nov. spec.* and the affinities and classification of spirochætes, *Quart. J. Micr. Sc.*, 1910–11, lvi, 507.
12. Zuelzer, M., Über *Spirochæta plicatilis* Ehrenberg und deren Verwandtschaftsbeziehungen, *Arch Protistenk.*, 1912, xxiv, 1.
13. Schellack, C., Studien zur Morphologie und Systematik der Spirochæten aus Muscheln, *Arb. k. Gsndhtsamte*, 1909, xxx, 379.
14. Gross, J., Ueber Systematik, Struktur und Fortpflanzung der Spironemacea, *Centr. Bakt., Ite Abt., Orig.*, 1912, lxv, 83.

EXPLANATION OF PLATES.

PLATE 21.

Magnification uniformly × 1,000.

FIGS. 1 to 3. Dark-field view of living specimens of *Cristispira balbianii* from an oyster. Photographed while they were temporarily at rest. The smooth wavy body with sharp contour along the wall, and a somewhat hazy alveolar content are well shown. The undulating crista loosely attached to the body of the organism can be seen. What appear like pointed sticks attached to the organism in Figs. 1 and 3 are the spirillar organisms found in fresh oysters which were merely entangled in the crista.

FIGS. 4 and 5. Dark-field view of *Cristispira balbianii* in the styles of oysters. In the styles there are different forms undoubtedly representing various stages of the life of the organism. In Fig. 4, there are one degenerated and two well preserved specimens, while in Fig. 5 there are also two intact and one long skeleton of a degenerated organism.

FIGS. 6 to 9. Different phases of degeneration of *Cristispira balbianii* due to unfavorable conditions or to age. Fig. 9 shows the protoplasm bulb accumulated near the middle of the body under the influence of a concentrated solution of saponin. Figs. 6 and 8 show the elementary fibrils which constitute the crista.

FIG. 10. Dark-field view of a cristispira from the style of a clam. In every respect this organism is comparable to the *balbianii* found in oysters. It may be the same as that described by Dobell as *Cristispira veneris*.

FIG. 11. Dark-field view of a cristispira from the style of a modiola. Generally somewhat longer than the *balbianii;* otherwise appears identical.

PLATE 22.

Magnification uniformly × 1,000.

FIGS. 12 to 15 and 17. *Cristispira balbianii* from oysters, stained with Giemsa's solution after osmic acid or sublimate alcohol fixation. They are all well preserved.

FIGS. 16, 18, and 19. *Cristispira balbianii* stained with Giemsa's solution after methyl alcohol fixation. All except Fig. 16 appear in good condition, although no definite cross-bar structure can be distinguished.

FIGS. 20, 21, and 23. *Cristispira balbianii* in process of degeneration. Fig. 20 shows the body still solid, almost intact, with a regularly wavy crista. In Fig. 21 the body is still fairly well preserved, but it follows the regular waves of the crista, indicating the absence of any resistance to the form of the crista. Note also the protruding mass of protoplasm near the middle of the body. In Fig. 23 there is only a shadowy trace of the body and the spiral elastic skeleton, or crista, is distinct.

FIG. 22. A section of the oyster style fixed in sublimate alcohol and stained with Mallory's iron-hematoxylin. The organisms show the chambered structure, but no crista can be distinguished.

FIGS. 25 and 27. *Cristispira balbianii* in films from fixation sublimate alcohol and stained with Heidenhain's iron-hematoxylin.

FIGS. 24, 26, and 28. Cristispira from a clam caught in New York Bay. The film was fixed in sublimate alcohol and stained with Heidenhain's iron-hematoxylin.

PLATE 23.

Magnification ×1,250.

Drawings from film preparations fixed in osmic acid vapor and absolute alcohol and stained with Giemsa's solution.

FIGS. 29 to 42. Different specimens of *Cristispira balbianii* showing the general chambered or cross-barred appearance of the body and undulatory membranous appendage, called the crista by Gross. The blue-stained substance which constitutes the cross-bars, or partitions, is seen to be regularly or sometimes irregularly distributed in amount or distance. In some, the substance may not be attached to the wall at all, but forms longitudinal rods of varying lengths as seen in Figs. 41 and 42. In such instances there is no suggestion of a chambered structure.

FIGS. 36 to 39. The final steps of degeneration of *Cristispira balbianii*. Fig. 36 alone still shows the degenerated body while the others show nothing but the fibrous skeletons, or cristas, of the organism. Fig. 39 is a fragment of the crista.

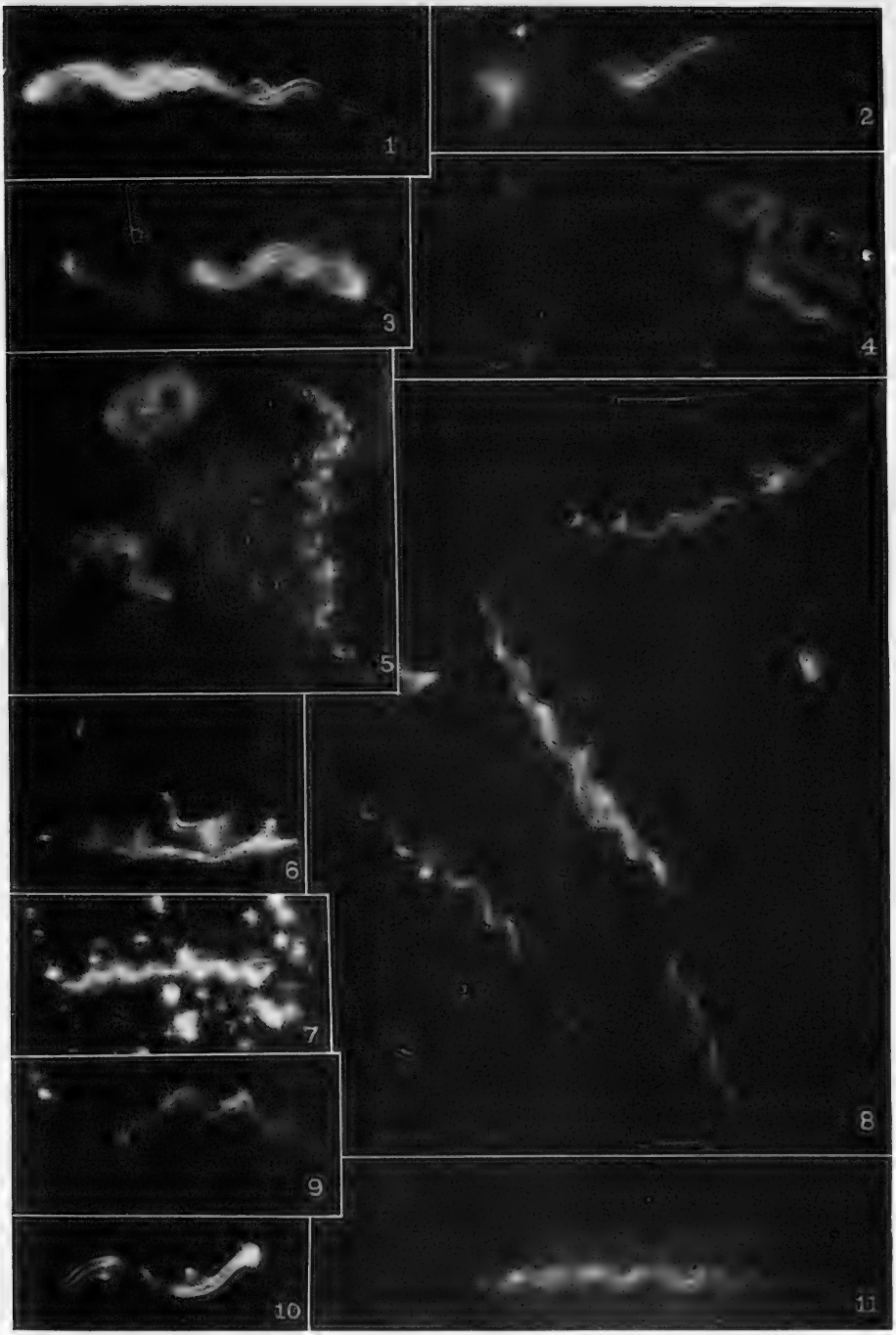
FIG. 43. The spiral organism found in the crystalline styles of fresh oysters caught in Woods Hole Bay.

PLATE 24.

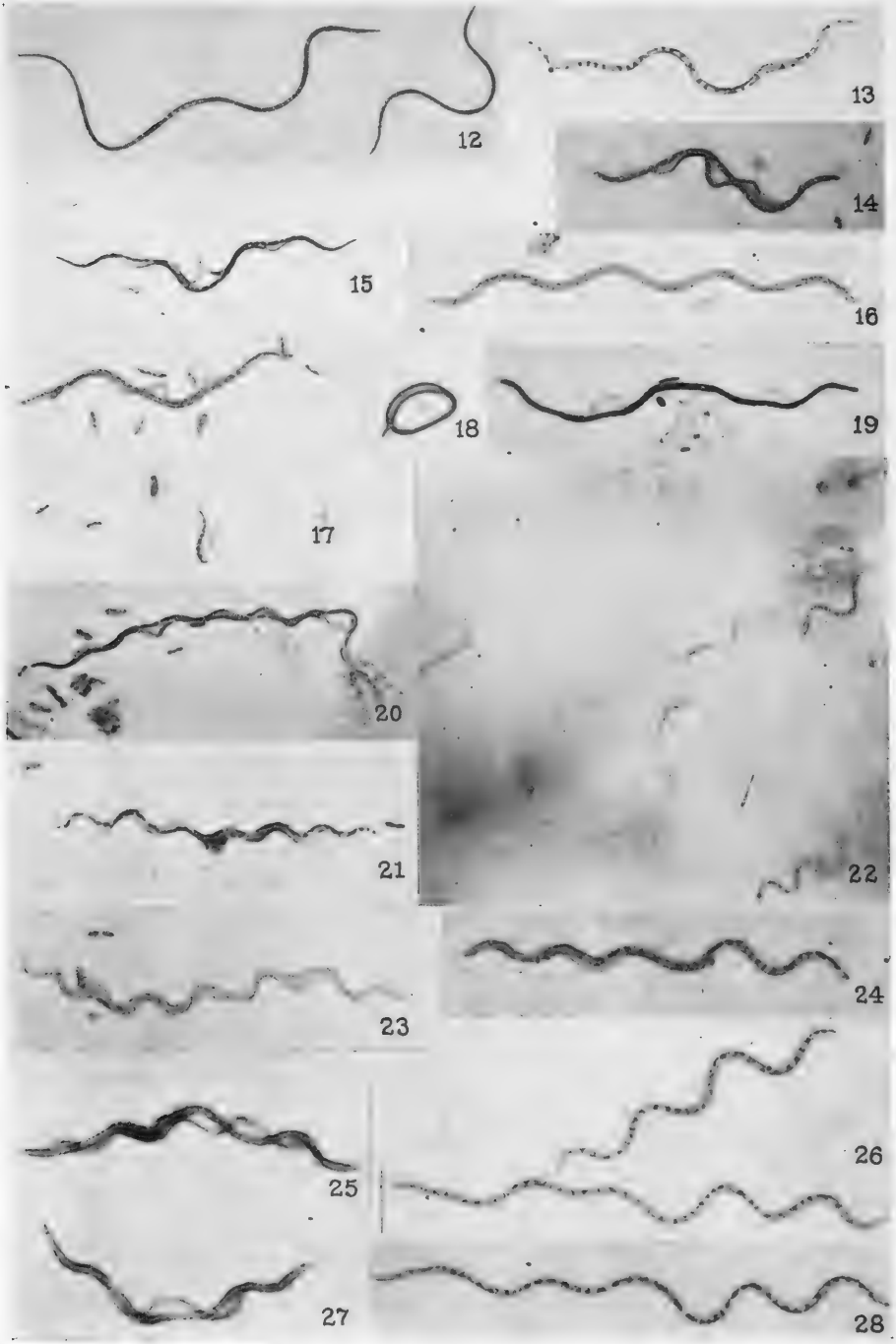
Magnification ×1,250.

FIG. 44. *Cristispira balbianii* from an oyster. Film preparation, fixed in sublimate alcohol and stained with Heidenhain's iron-hematoxylin.

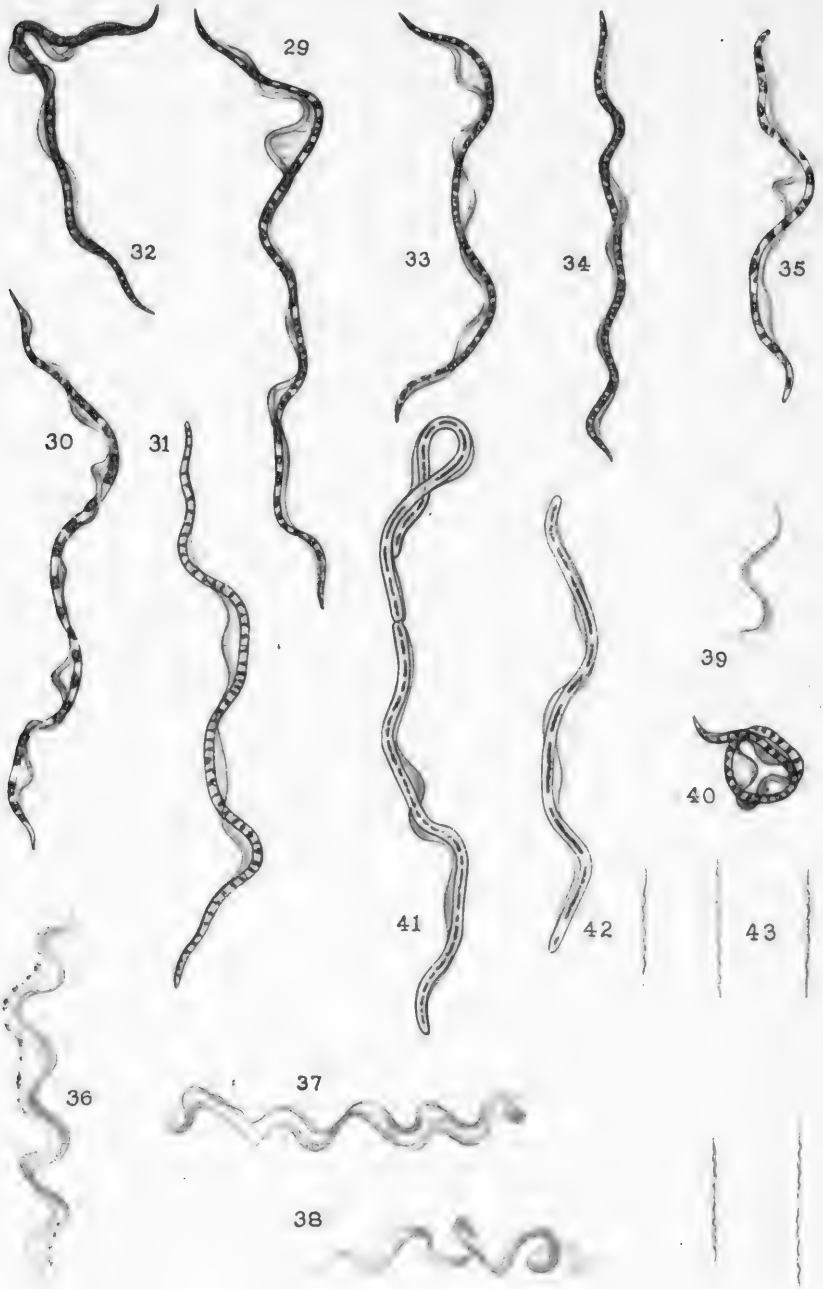
FIGS. 45 and 46. A cristispira (*Cristispira veneris?*) from a clam collected near New York Bay. Sublimate alcohol fixation and Heidenhain's iron-hematoxylin.



(Noguchi: *Cristispira* in North American shellfish.)



(Noguchi: *Cristispira* in North American shellfish.)



(Noguchi: *Cristispira* in North American shellfish.)

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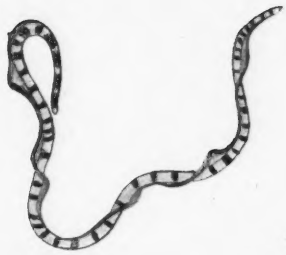

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(Noguchi: *Cristispira* in North American shellfish.)